

Science in the Arab world

The immense challenges facing those who attempt to support research in developing countries are compounded by political turmoil in the Middle East.

Four years ago, *Nature* reported on some welcome news from the Arab world. After years of declining investment in research, Arab states had begun to spend more on science and technology. The increases were modest and began from a low base, but they were happening in many countries that, owing to low oil prices, were experiencing falling national incomes at the time.

At the same moment, a new pan-Arab funding and networking organization had emerged. The Arab Science and Technology Foundation (ASTF), based in Sharjah in the United Arab Emirates — one of the region's most dynamic territories — aimed to function much like a national science-funding agency, offering grants to scientists based on an open, competitive process and peer review (see *Nature* **416**, 120–122; 2002).

Nothing Earth-shattering here, you might think — except that in many developing countries, such agencies are under-funded, overly bureaucratic, and lacking in transparency and the in-house capacity to judge quality. It was partly to avoid these pitfalls that the ASTF set itself up as a non-profit foundation at arm's length from governments, instead seeking finance from the private sector. The foundation also aimed to encourage expatriate Arab scientists in Europe and the United States to work with scientists from home, and to link investors with researchers, holding workshops on topics such as entrepreneurship and intellectual property.

The importance of such activity cannot be overstated. The countries of the Middle East and North Africa are near the bottom of the global league tables for patents and trademarks. Foreign investment into, and high-technology exports from, Arab nations are also among the lowest in the world. As more Arab nations join the World Trade Organization — Saudi Arabia is the latest to do so — the ASTF wants the region's scientists to help their nations compete in global markets.

Changing relationships

The foundation's grants programme is now established in fields such as water desalination, cellular telephony, materials science, robotics and biomedical research. And the organization has grown more ambitious. For example, there was no meeting where the Arab world's scientists could get together and learn about each others' work. So the ASTF started one: the biennial Scientific Research Outlook Symposium. The 2004 event in Riyadh, Saudi Arabia, attracted 850 scientists, twice the number of the previous meeting.

Unusually for a scientific body, the ASTF has engaged in difficult public-policy issues, including one of the region's biggest problems: the future of Iraq. Despite widespread scepticism in the Arab world about the invasion, and the murder of many scientists in Iraq (see page 1036), the foundation is working with the country's government to help reorganize science there.

This month, the ASTF announced its most ambitious programme

to date: Izdihar, which is Arabic for prosperity. The programme's aim is to raise quality of life in Arab societies, partly through the commercialization of knowledge. Under Izdihar, the ASTF promises to develop research-based solutions for problems in agriculture, energy, the environment and health care — and even governance.

Izdihar's designers argue that the region's problems are so severe that the ASTF's current activities, no matter how successful, are unequal to them. What is needed, they believe, is a strategic vision with science, technology and commerce at its core. The project is a big idea, and the result of Arab scientists' belief that they are privileged and therefore must shape a better future for those less fortunate. What its designers must now do is to explain how they intend

"In seeking to harness research and enterprise to alleviate poverty and underdevelopment, the Izdihar project demonstrates both vision and a sense of civic responsibility."

to realize this vision. What can the concept deliver, and at what cost?

Such an explanation is needed in a region where other plans to revive science and technology have withered. Take the most recent of these: a planned multinational research fund that

would span the Muslim world (see *Nature* **432**, 273–274; 2004). The fund would see science spending set aside and administered by a group of international trustees, making it hard for governments to spend the money on other things.

The scheme was championed by Pakistan's President Pervez Musharraf, and endorsed by the science ministers of the 57 member states of the Organization of the Islamic Conference. But it has failed to excite the governments that would have paid for a lot of it, such as those of Kuwait and Saudi Arabia. Without their support, the fund will probably be stillborn.

In contrast to this pattern of unfulfilled promise, the ASTF has worked hard to show that it means what it says. It offers hope in a realm of pervasive pessimism. And in seeking to harness research and enterprise to alleviate poverty and underdevelopment, the Izdihar project demonstrates both vision and a sense of civic responsibility.

But Izdihar is unlike anything that the foundation has embarked upon before. The organization's supporters — including the Arab investors and donors who would pay for the project and the scientists who would do the work — will have to be convinced that the foundation can deliver. The ASTF must therefore show not just a clear grasp of the problems, but also that it understands their solutions, and that it can mobilize the necessary resources. That will be a challenge, even for an organization that has shown so much early promise. ■



A tale of one world

Celebrating science on the solstice.

It was the best of times, it was the worst of times. Thus wrote Charles Dickens at the start of *A Tale of Two Cities*, before running through a sequence of superlatives and concluding it to be “a time like any other”. Its salient feature was that it was the day on which Dickens wanted to begin his tale.

The same could be said of our decision to publish, in this week's *Nature*, a moment-by-moment account of one day in the world of science (see page 1040; or for greater detail our website). The day is 21 June, the summer solstice. It is the longest day; it is the shortest day. It is the most ancient day (marked by humankind with its earliest megaliths) and the most recent day (give or take the subsequent seven). But it is, in the end, a day like any other.

And therein lies the wonder. It is an ordinary day filled with extraordinary goings-on. It is easy for scientists working on their own projects to forget the breadth, complexity and intricacy of the overarching project of which they are part, the project of understanding the world and its contents. Concentrating on one day, from the seas off Singapore and Svalbard to the mountains of Madagascar and the ice-cap of Antarctica, from glimpses of the farthest galaxies to the afterglow of a strangely close gamma-ray burst, is a way of celebrating that quest for knowledge in a day's worth of arbitrary details.

And in every detail there is a human story. People finish projects

and begin them (although most are in the middle, as most things are, most of the time). People receive good news — that a vaccine has been approved — and bad — in the unexpected shortcomings of a diagnostic test. Children learn ancient knowledge (how to measure the circumference of the Earth) and modern know-how (how to race a toy car powered by a fuel cell). People offer small kindnesses and share global enthusiasms.

But what we are celebrating here is not just on an individual scale. It is on the scale of the planet. By taking the whole world as its subject, science pulls the world together, whether by cloning rare forest oxen or assessing the vulnerability of arctic algae to ultraviolet light, by mapping undersea currents or comparing infinitesimally precise measurements made continents apart to sense the expansion and contraction of space.

Science, perhaps more than any other human endeavour, stretches around and through the Earth. It reaches places where commercial activity is banned, such as the South Pole, and where politics are meaningless, such as the night sky, aglow with the faint passage of cosmic rays. It penetrates into minds deciding between breakfast cereals on supermarket shelves and pools the whole world's exhalations — animal, vegetable and internally combusted mineral — into a single measurement of the state of the atmosphere on a mid-ocean mountaintop.

While nature never fails to inspire, the process of understanding it can sometimes efface itself. Yet the process is wonderful and remarkable, and last Wednesday, many of you played a part in it. It was a day, like any other — and thus worthy of celebration. ■

Action stations

The time for sitting on flu data is over.

Concern about the accessibility of data on flu strains remains an acute issue, which research administrators and political leaders should step forward and address.

Indonesia has become the hot spot of avian flu, with the virus spreading quickly in animal populations, and human cases occurring more often there than elsewhere. Yet from 51 reported human cases so far — 39 of them fatal — the genetic sequence of only one flu virus strain has been deposited in GenBank, the publicly accessible database for such information.

And last week in China, researchers belatedly published details of a case that tested positive for the virulent H5N1 strain in 2003 — contradicting the government's official line that none had occurred before November 2005. The unnecessary delay reaffirms the critical importance of better dissemination of flu data.

Back in Indonesia, the World Health Organization (WHO) has just confirmed that a cluster of eight cases in an extended family in northern Sumatra was the first unequivocal occurrence of limited human-to-human transmission of the virus. Whereas the WHO initially stated that the virus in the cluster showed “no significant” mutations, it now says that genetic changes in the virus account for

the appearance of human-to-human transmission. In the Sumatra event, the transmission did not spread beyond the family.

Yet scientists outside the WHO networks have no access to these data. The problem last year spurred the US National Institutes of

“H5N1 sequences should be promptly deposited in a publicly accessible database.”

Health (NIH) to create a consortium to sequence and make public thousands of flu strains from humans and birds. Very quickly, this more open approach led to the useful discovery that viruses swap genes with each other more

frequently than had been previously thought.

Some political leaders are drawing the appropriate conclusions. Dennis Kucinich (Democrat, Ohio) and Wayne Gilchrest (Republican, Maryland) are circulating a letter in the House of Representatives that calls on Michael Levitt, the US health secretary, to require H5N1 sequences and other publicly funded research data “to be promptly deposited in a publicly accessible database, such as GenBank”.

An appropriate model for better data access is at hand. Earlier this month, the Paris-based World Organisation for Animal Health and the United Nations Food and Agriculture Organization said that they would work with the NIH to sequence H5N1 samples from birds and deposit them in GenBank. The WHO and its member states urgently need to establish a similar mechanism to ensure that data on human cases are immediately put in the public domain. ■

RESEARCH HIGHLIGHTS

Up for a fight?

Am. Nat. **168**, 100–113 (2006)

The wide-open mouth of an adult male collared lizard (*Crotaphytus*; pictured) signals not amazement, but aggressive intent. Such 'gaping' is a feature of male-male displays. A. Kristopher Lappin of Northern Arizona University in Flagstaff and his colleagues have looked into what exactly is on show.

The main weapon of these lizards is their bite — about which contenders might want information if they are to decide whether or not to join battle. From measurements of bite force and of the jaw muscles inside the mouth, Lappin and his co-workers conclude that the size of the muscles, shown during gaping, provide a bite-force indicator. Hence the display — the message of which, the authors suggest, is amplified by conspicuous white patches at the corners of the lizard's mouth.



A. K. LAPPIN

SEISMOLOGY

Danger spotting

Geophys. Res. Lett. **33**, L11309 (2006)

The devastating tsunami in the Indian Ocean on 26 December 2004 could have been anticipated even without a dedicated early-warning system, according to Geoffrey Blewitt of the University of Nevada in Reno and his co-workers.

They show that ground movements of 1 centimetre or more in India — about 2,000 kilometres from the epicentre of the magnitude-9 earthquake that caused the tsunami — could have been detected by the Global Positioning System (GPS) just 15 minutes after the start of the quake, and used to calculate the true size of the event. Existing seismic sensors initially indicated that the quake was of magnitude 8–8.5, which was considered too small to generate an oceanwide destructive tsunami. These inaccurate estimates meant that warning of the tsunami hazard was delayed by hours.

MARINE BIOLOGY

Chain reaction kills coral

Ecol. Lett. **9**, 835–845 (2006)

Researchers in the United States provide new insight into a chain reaction that is killing the world's reefs.

Jennifer Smith, now of the University of California, Santa Barbara, and her colleagues reveal how algae — which increase when overfishing depletes the natural grazers that

control the marine plant — can contribute to coral damage. They find that the algae release sugars that fuel the growth of coral-suffocating bacteria.

The team performed experiments using the green algae *Dictyosphaeria cavernosa* and *Pocillopora verrucosa* coral that was recovered from Palmyra Atoll, about 1,750 kilometres south of Hawaii in the Pacific Ocean.

CLIMATE SCIENCE

Poles apart

Science doi:10.1126/science.1123296;

10.1126/science.1125249 (2006)

Help may be at hand for climate scientists struggling to explain dramatic shifts in Earth's past ice cover.

The periods of glaciation that began around 3 million years ago come and go on a 40,000-year cycle, which clearly follows changes in Earth's tilt towards the Sun. What puzzled scientists was the lack of evidence for a cycle tracking the planet's proximity to the Sun during summer, which varies over a 20,000-year period. These properties of the Earth and its orbit affect the amount of sunshine reaching Earth's surface, which in turn influences ice cover.

Maureen Raymo of Boston University and her colleagues argue that the proximity effect is cancelled out in the sea-level records used to track glaciation

because changes in ice volume at the South Pole are equal and opposite to those at the North Pole. In a separate study, Peter Huybers of Harvard University in Cambridge, Massachusetts, suggests instead that proximity is simply cancelled by speed: when Earth is close to the Sun, it moves faster, so a hot summer is also a short one.

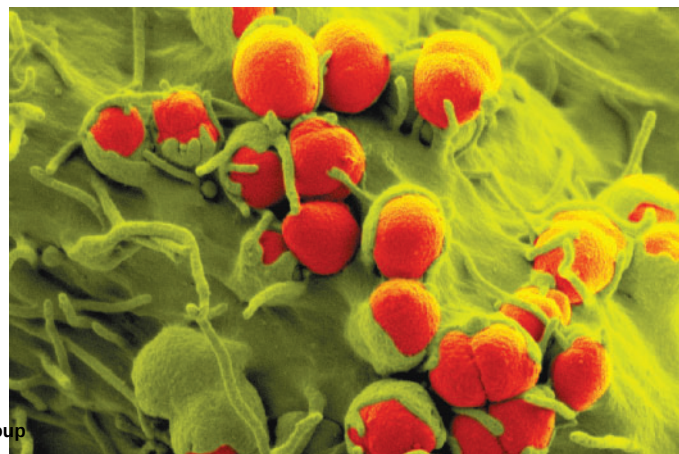
The two hypotheses have different implications for global climate; further data might determine which effect is the greater.

MICROBIOLOGY

Quick costume change

PLoS Biol. **4**, e185 (2006)

Neisseria gonorrhoeae (pictured below), the bacterium responsible for the sexually transmitted disease gonorrhoea, manages to thwart the body's immune system by rapidly changing its outer proteins, or pilins. Researchers suggest that the bug might



SPL

accomplish this trick by having more than one genome copy per cell.

Hank Seifert and Deborah Tobiason of Northwestern University, Chicago, Illinois, discovered through flow cytometry and other molecular-biology techniques that *N. gonorrhoeae* has on average three genome copies per cell, with some cells having two and others up to six. This would make it easier for the bug to alter its proteins because it can shuffle bits of pilin gene between its genomes.

GENETICS

Director's cut

Nature Meth. **3**, 503–509; 511–518 (2006)

For more than 25 years, researchers have identified genetic control regions, such as promoters, by virtue of the fact that they are in accessible parts of the chromosome and can be cut by the enzyme DNase I.

Now two groups have mapped all such DNase I-hypersensitive sites in 1% of the human genome. Francis Collins of the National Human Genome Research Institute in Bethesda, Maryland, and John Stamatoyanopoulos of the University of Washington, Seattle, and their colleagues let the enzyme digest human DNA, then put the fragments on microarrays to pinpoint the cuts.

The papers show that the regulatory regions differ from one cell type to another, that they cluster around the ends of genes, and that they are organized into 'superclusters' spanning hundreds of thousands of base pairs.

CELL BIOLOGY

Pore relations

Nature Cell Biol. doi:10.1038/ncb1424; doi:10.1038/ncb1427 (2006)

Messenger RNAs are produced in the nucleus, but are used to make protein in the cytoplasm. Two studies shed new light on how they cross the nuclear membrane.

Karsten Weis of the University of California, Berkeley, and Susan Wente of the Vanderbilt University Medical Center in Nashville, Tennessee, and their colleagues focused on the role of the protein Dbp5, which attaches to newly synthesized RNAs.

The teams show that Dbp5 is activated by binding to another protein, Gle1, and a small cofactor known as InsP₆. Gle1 sits on the cytoplasmic face of the nuclear pore complex that allows transport across the nuclear membrane. What Dbp5 is doing is unclear, but the authors speculate that its activation at this point might ensure that mRNAs make it through the pore and do not go back.

ASTRONOMY

Are we alone?

Astrophys. J. **644**, 1223–1231 (2006)

Are there habitable planets of Earth mass outside the Solar System? Observationally, we can't say — current techniques are unable to find extrasolar planets that small.

Sean Raymond, now at the University of Colorado in Boulder, and his colleagues sidestep this difficulty by performing multiple simulations of planet formation in four solar systems known to contain gas-giant planets similar to Jupiter.

Their results are not terribly encouraging. If the giant planets formed early and migrated quickly through the zone of terrestrial-planet formation, one of the four systems developed a promising candidate. This weighed in at 0.6 Earth masses and contained a substantial water fraction, some of which would be liquid.

But if the giant planets migrated later, they shook up the planets' cradle so much that no system conceived a habitable planet.

NEUROBIOLOGY

Make or break

Nature Med. doi:10.1038/nm1438 (2006)

A new way of measuring protein turnover in the human central nervous system could be used to probe the roots of diseases such as Alzheimer's.

Neurodegeneration in Alzheimer's disease is associated with the accumulation in the brain of the protein amyloid- β . This could be caused either by enhanced production of the protein by neurons, or by a disturbance in its clearance. Randall Bateman of the Washington University School of Medicine, St Louis, Missouri, and his colleagues have developed a technique to clock these rates *in vivo*.

They measured the uptake of an isotope-labelled amino acid into amyloid- β protein as it was being synthesized. In healthy patients, uptake roughly matched the protein's breakdown rate. An imbalance may provide an indicator of disease.



NASA

JOURNAL CLUB

Peter Moore
King's College London, UK

An ecologist finds fault with fecundity.

I am disturbed both by the current pace of environmental change, and by the lack of sound survey data that allow the biological impact of such changes to be measured.

Vegetation responses to change are generally slow, and so it takes decades of committed survey work to document them effectively.

Such research is often neglected because it generates few publications per unit of effort and time expended.

Fortunately, a few far-sighted ecologists, working in the days when science was assessed by quality and need, laid foundations upon which we can build. Among these was Franklyn Perring, a skilled and devoted field botanist, who spent the summers of 1952 and 1953 surveying the chalk grasslands of England.

What I admire most about his work is his precise and accurate recording of site locations and methods. This made it possible for others to repeat his survey half a century later (J. Bennie *et al. J. Ecol.* **94**, 355–368; 2006).

Chalk grasslands are in steep decline as a result of changes in land use. Even the nitrogenous compounds in air pollution represent a subtle threat. Their input increases soil fertility, encouraging aggressive grasses at the expense of the sensitive, slow-growing plants typical of chalk habitats. In our patchwork planet, fragments of chalk grassland are also at risk of invasion by the productive species around their extended edges.

The new survey shows that only the toughest, most inhospitable sites with steep slopes, shallow soils and south-facing aspects have effectively resisted the seemingly unstoppable advance of fecundity.

This publication should ring alarm bells about the need to fund long-term monitoring programmes in all threatened habitats.

NEWS

Academy affirms hockey-stick graph

IPCC

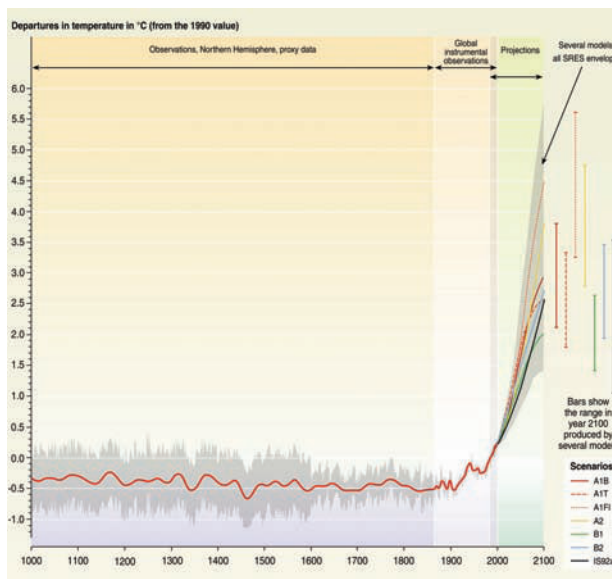
WASHINGTON DC

It's probably the most politicized graph in science — an icon of the case for climate change to some, and of flawed science in the service of that case to others — and it has coloured the climate-change debate for nearly a decade. Now the US National Academy of Sciences (NAS) has weighed in with a report on the 'hockey-stick' plot, which it hopes will finally lay the controversy to rest.

The graph purports to chart global temperatures over the past millennium; a sharp rise at the current end is the 'blade' that makes the otherwise flattish line look like a hockey stick. Climate groups have claimed it as evidence of dangerous global warming; sceptics, especially in the United States and Canada, have questioned the study's merit and statistical methodology.

In its report, released on 22 June, the NAS committee more-or-less endorses the work behind the graph. But it criticizes the way that the plot was used to publicize climate-change concerns. And it leaves open big questions about whether researchers should be obliged to make their data available (see 'Plotting a course').

"We roughly agree with the substance of their findings," says Gerald North, the committee's chair and a climate scientist at Texas A&M University in College Station. In particular, he says, the committee has a "high level of confidence" that the second half of the twentieth century was warmer than any other period in the past four centuries. But, he adds, claims for the earlier period covered by the study, from AD 900 to 1600, are less certain. This earlier period is particularly important because global-



Cause for controversy: Michael Mann used proxies for climate change, such as tree rings, to produce a picture of Earth's changing climate over the past millennium.

T. COGHILL

warming sceptics claim that the current warming trend is a rebound from a 'little ice age' around 1600. Overall, the committee thought the temperature reconstructions from that era had only a two-to-one chance of being right.

The graph arose from the work of Michael Mann, a climatologist now at Pennsylvania State University in University Park, and two colleagues. In two papers published in 1998 and 1999, Mann's team examined tree rings, ice cores and other 'proxies' of past climate, and used them to reconstruct the Northern Hemisphere's temperature over the past millennium (M. E. Mann *et al.* *Nature* **392**, 779–787; 1998

and M. E. Mann *et al.* *Geophys. Res. Lett.* **26**, 759–762; 1999).

The analysis was complex because the proxies were geographically dispersed and contained uncertainties that are often difficult to gauge. For example, the growth of bristle-cone pine trees, which played an important role in the Mann study, depends on temperature, but also rainfall. The researchers concluded in their 1999 paper that "the 1990s are likely the warmest decade, and 1998 the warmest year, in at least a millennium", and included a graph showing a sharp upturn in temperature from about 1900 onwards. The plot soon became

PLOTTING A COURSE

The US National Academy of Sciences (NAS) report may help put the 'hockey-stick' debate to rest, but it leaves open a larger question about who should have access to researchers' data.

Mann's critics complained that he would not share the methodological details or raw data that would enable them to assess his work. In June 2005, congressman Joe Barton (Republican, Texas) wrote to the hockey-stick study's authors

demanding that, among other things, they list their funding agencies and provide access to their raw data and algorithms. Letters were also sent to the National Science Foundation and the Intergovernmental Panel on Climate Change demanding information on the work (see *Nature* **436**, 7; 2005). Many in the scientific community were shocked by the letters' aggressive tone, but Mann did supply information about how to access the data he used.

Barton and his committee have commissioned their own statistical analysis, which is ongoing.

The NAS panel largely demurred on questions of data sharing, saying that although openness is generally good, such issues are "discipline dependent". "We thought that question was a little big for what we were trying to do here," says committee chair Gerald North.

That doesn't mean the matter is closed. Bill Kearney, the academy's director of media relations, says

that the NAS is putting together a panel to examine issues of data sharing in all fields. The panel, which will convene later this summer, "will be a broad look at access across the board", says Kearney.

For now, politicians will probably intervene as they see fit, says David Goldston, chief of staff for the House Committee on Science: "As the scientific community continues to wrestle with this, it will continue to be an issue."

G.B.

known as the hockey stick, and was featured prominently in the executive summary for policy-makers in the 2001 report on global warming from the Intergovernmental Panel on Climate Change (IPCC).

Shortly after it appeared in the report, two Canadians, economist Ross McKittrick and mineral-exploration consultant Stephen McIntyre, attacked the methodology behind the graph, claiming that it was based on insufficient data and flawed statistical analysis. US politicians amplified their complaints, most prominently Representative Joe Barton (Republican, Texas), who in 2005 wrote to Mann demanding he share his data with critics and congressional overseers. In an effort to quell the controversy, the chairman of the House Committee on Science, Representative Sherwood Boehlert (Republican, New York), commissioned the academy to examine the earlier work.

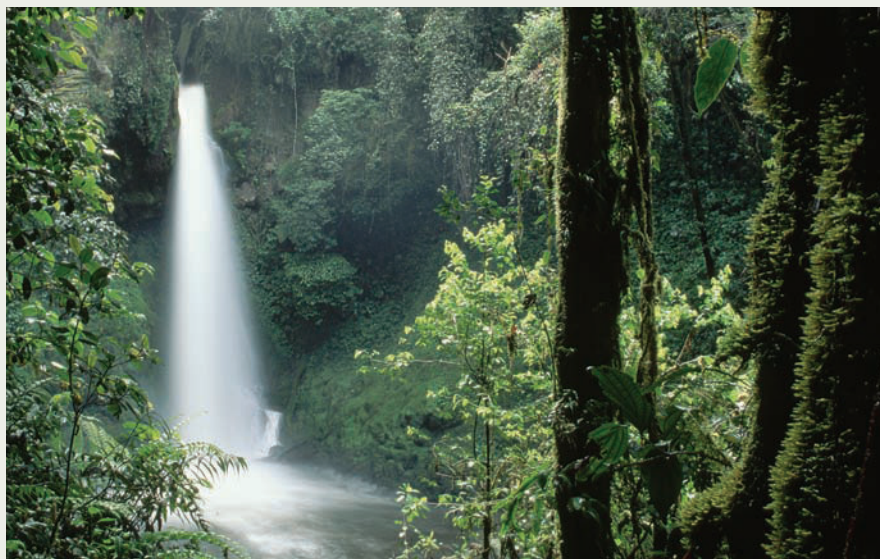
The academy essentially upholds Mann's findings, although the panel concluded that systematic uncertainties in climate records from before 1600 were not communicated as clearly as they could have been. The NAS also confirmed some problems with the statistics. But the mistakes had a relatively minor impact on the overall finding, says Peter Bloomfield, a statistician at North Carolina State University in Raleigh, who was involved in the latest report. "This study was the first of its kind, and they had to make choices at various stages about how the data were processed," he says, adding that he "would not be embarrassed" to have been involved in the work.

Panel members were less sanguine, however, about whether the original work should have loomed so large in the executive summary of the IPCC's 2001 report. "The IPCC used it as a visual prominently in the report," says Kurt Cuffey, a panel member and geographer at the University of California, Berkeley. "I think that sent a very misleading message about how resolved this part of the scientific research was."

"No individual paper tells the whole story," agrees North. "It's very dangerous to pull one fresh paper out from the literature."

Mann says that he is "very happy" with the committee's findings, and agrees with the core assertion that more must be done to reduce uncertainties in earlier periods. "We have very little long-term information on the Southern Hemisphere and large parts of the ocean," he says. As for the report's effect on the policy debate, Mann says: "Hopefully this is the beginning of us, as a community, putting that silliness behind us." ■

Geoff Brumfiel



W. KAEHLER/CORBIS

Experts comb tropics for clues to vCJD

Some people in Papua New Guinea who once feasted on their own relatives did not succumb to the prion disease kuru until 50 years later, say researchers who have laboriously tracked down the last sufferers in remote villages. The discovery renews concern that another human prion disease, variant Creutzfeldt-Jakob disease (vCJD), might be incubating silently in some populations and could rear its head decades from now.

Neurologists have long been fascinated by kuru, which caused an unprecedented epidemic of neurodegenerative disease in the Fore people of Papua New Guinea that peaked in the 1950s and early 1960s. In death rituals, families steamed and ate the bodies of their relatives — along with, it was later discovered, infectious prion proteins that caused the debilitating and fatal disease. The ritual was prohibited in the mid-1950s by the Australian authorities who governed that part of Papua New Guinea, and the disease eventually became less frequent.

Interest in kuru reawakened with the realization that vCJD,

transmitted from cows infected with bovine spongiform encephalopathy, might cause a similar epidemic among those who ate infected meat in the 1980s and 1990s. So far, only 156 deaths from vCJD have been reported in Britain, the worst-affected country, and the number of new cases peaked in 2000, suggesting that vCJD takes about ten years to incubate. But debate continues about whether another wave of cases could yet appear.

John Collinge at University College London and his colleagues went to Papua New Guinea to find out. Most people with kuru have already died, but the team ramped up existing disease monitoring to find the last of the epidemic. Working with local communities, they scoured isolated villages that are typically more than 2,000 metres above sea level, lost in dense rainforest and connected only by tracks. "It's arduous trekking," says Collinge.

Between 1996 and 2004, the researchers found what they believe are the last 11 cases of kuru. Patients' histories were collected, to piece together when they were probably

infected. The longest incubation time was calculated to be at least 56 years, and perhaps seven years longer — although the average incubation time seems to be 12 years (J. Collinge *et al. Lancet* 367, 2068–2074; 2006). "For the first time we can see the extraordinary incubation period in human prion disease," says Collinge. "It's sobering that, half a century on, this disease has not disappeared."

Collinge says that vCJD could have a much longer average incubation time, of 30 years or more, because the prions are passing from cows to humans rather than between humans. A species barrier extends incubation times in animal tests. People who have already succumbed to vCJD might have been particularly genetically susceptible, as other evidence has suggested.

Mathematical models used to predict the size of a vCJD epidemic could now include these findings. "Most people seem to think we're over the worst," Collinge says. "We have to be cautious about assuming this disease is going away." ■ Helen Pearson

ON THE RECORD

“This was the only time the United States has beaten Brazil in anything soccer-related.”

An observer at this year's RoboGames, in San Francisco, comments on the US triumph in a football match played by humanoids.

“In the showers afterward, they pretty much knew which pill they had been given.”

Physiologist Anne Friedlander describes a study in which some competitive cyclists took sugar pills, as a control group for high-altitude cycling studies. The others got Viagra.

Sources: *Wired News, Chicago Tribune*

SCORECARD**Colourful sport**

Football uniforms aren't just pretty: psychologists say they help spectators track more than three players at a time.

**Clowns**

In vitro fertilization works better if women are entertained by trained clowns after their treatment, according to an Israeli study.

**Future of energy**

The International Energy Agency suggests that the world's energy needs will more than double between 2003 and 2050 — and coal will still be the top power source.

NUMBER CRUNCH

Twenty-eight years have passed since the first child was born through assisted reproduction. Now, women around the world are lining up to try for test-tube babies.

3 million babies have been born through assisted reproduction since 1978.

200,000 babies were born through it in 2002 alone.

3.9% of all Danish babies are born using assisted reproduction techniques — the highest proportion in the world.

Source: 2002 *World Report of the International Committee for Monitoring Assisted Reproductive Technologies*.

Hungary's science academy slammed as 'obsolete'

Hungary's national science academy has been criticized for discriminating against scientists living and working abroad. The academy's attitude is frustrating not just researchers but also the Hungarian government, which is trying to reform the country's research system and attract more high-profile scientists.

The Hungarian Academy of Sciences (HAS) in Budapest is Hungary's largest and best-funded public research institution. It awards a

title, 'Doctor of Science', that is required by professors or lecturers at most Hungarian universities and academic research institutes. Applicants need a certain number of scientific publications, but *Nature* has learned that the academy's medical division treats non-Hungarian publications as worth only half as much as those published in Hungary.

"The rules basically exclude foreign researchers from competition with medical sci-

The Hungarian Academy of Sciences is accused of putting scientists who publish abroad at a disadvantage.



L.JANICEK/ZEFA/CORBIS

Hunt for AIDS vaccine tackles genomes

A coalition of scientists aims to kick-start the stalled search for a vaccine against the human immunodeficiency virus (HIV). The researchers will mine the genomes of HIV-positive patients from many countries in the hope of finding ways to help the immune system fight HIV.

Scientists have been trying to figure out for years why some people are less susceptible to HIV than others. Studies on specific groups of patients such as Kenyan prostitutes have identified a handful of natural

genetic variations that influence susceptibility to the infection. Leaders of the coalition now plan to combine data from many of these groups, to scan the whole genome of each patient instead of particular genes, and to focus on patients who have been tracked since the early days of their infection.

"The past 10 or 15 years of genetic work on HIV cohorts has been pretty painful," says Amalio Telenti of the University of Lausanne in Switzerland, an organizer of the initiative. "This

is a whole new ball game."

The coalition, called EuroCHAVI, will include data from 600 patients in seven European countries and Australia, and will be funded by the Center for HIV/AIDS Vaccine Immunology (CHAVI), based at Duke University in Durham, North Carolina. CHAVI investigators also plan to set up study groups in Africa.

The scientists will look for differences between patients at hundreds of thousands of specific locations in the

entists in Hungary," says Gábor Vajta, a Hungarian embryologist and cloning expert working at the Danish Institute of Agricultural Sciences in Tjele. He says he has no intention of returning to his native country. "But if I did, I would practically have to start from the very beginning."

"It's outrageous," agrees Csaba Szabó, a pharmacologist who, after ten years in the United States and United Kingdom, returned to Hungary last year.

The policy is also against the spirit of Hungary's membership of the European Union, says Georges Bingen, who oversees mobility programmes at the European Commission's Directorate General for Research. "This looks like a severe obstacle to mobility," he says. Last year, the commission set up a mobility charter for researchers in Europe, calling for countries to encourage researchers to work abroad. But the commission has no authority to force a scientific institution to do so.

The Hungarian government, which wants to strengthen Hungarian science, is also concerned. "Some of the academy's rules clearly disadvantage scientists who live and publish abroad," says János Kóka, the Hungarian minister responsible for science.

Kóka says the practice is symptomatic of the academy's old-fashioned attitude. "Its election committees still consist of academicians who were socialized in a totalitarian regime," he says. "They're used to spending tens of millions of euros without producing any results worth mentioning." The HAS spends a large portion of its budget on "inherited merits and obsolete institutions," Kóka says.

"They're used to spending tens of millions of euros without producing any results worth mentioning."

Norbert Kroó, the HAS's vice-president in charge of foreign relations, counters that the academy has been reformed since the fall of Hungary's communist government in 1990. Staffing has been cut by 40%, he says, and about 170 new research groups have been selected by peer review. The HAS promotes researchers' mobility across borders and between academia and industry, he adds. He says he wasn't aware of the discriminatory rules, and that he'll ask the medical section to take action: "If these rules really are applied they need to be changed."

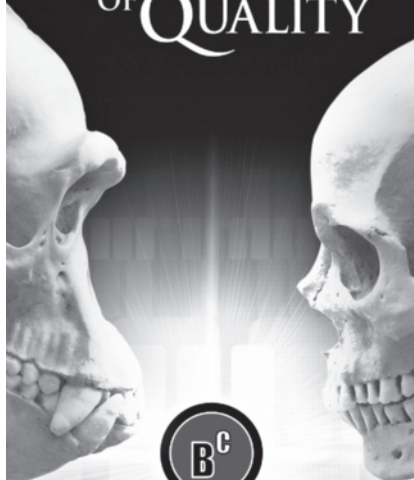
"There are two major lobbies within the academy," says Gábor Támas, a neuroscientist at the University of Szeged. "One faction wants changes, the other does not."

Támas says reform is needed urgently. But he warns against dismissing the academy's performance. Some HAS institutes, such as the Institute of Experimental Medicine in Budapest and the Biological Research Center in Szeged, produce some of the best science in the country, he says.

Critics and supporters of the academy should stop blaming each other, says Ernő Duda, president of both Solvo Biotechnology in Budapest and the Hungarian Biotechnology Association. He agrees that the academy needs to change, but also that Hungary's overly hierarchical universities must open up. "Until a few years ago I would have said that financing was the biggest obstacle to biotechnology in Hungary," he says. "Now the biggest problem is our obsolete and old-fashioned academic research system." ■

Quirin Schiermeier

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A. PASIEKA/SPL



Genome analysis might spot genes related to HIV response.

patients' genomes, hoping to find variations that influence how well people control the virus early in their infection. Identifying the genes involved should yield clues to how to design a better vaccine.

Large genetic studies are already under way for other conditions, including malaria and tuberculosis. But such an approach is more difficult with HIV because it cannot be studied in entire families, making it much harder to track inherited variations. The new studies have only become possible because of the recent construction of the HapMap, a catalogue of sites of

variation in the human genome that was published last year (International HapMap Consortium *Nature* 437, 1299-1320; 2005).

Even so, it's still a gamble that the whole-genome studies will yield results. "There's no guarantee that this approach is going to work — it's very much a betting game, like anything else we do," says Sunil Ahuja, a clinical researcher at the University of Texas Health Science Center in San Antonio, who is not involved with the EuroCHAVI study. "But you've got to take a shot." ■
Erika Check

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SPECIAL REPORT

Scientists become targets in Iraq

Violence is common currency in Iraq, but one group is increasingly and persistently singled out — academics.

Declan Butler reports on the risks run by researchers as they struggle to pursue their studies.

A chemist's mutilated body dumped on a street in Basra; a physicist shot twice in the back in Baghdad; a dean of engineering kidnapped by a hit squad, his body left on his wife's doorstep. Each week brings reports from Iraq of assassinations or kidnappings of scientists, academics and intellectuals, in what many argue is a systematic effort to eliminate or exile a group crucial to the country's reconstruction.

One of the first academics murdered was Muhammad al-Rawi, president of Baghdad University, assassinated in his clinic by a hit squad on 27 July 2003. In the chaos of Iraq, precise body counts are impossible, but observers have recorded several hundred assassinations of academics, with the rate of killings increasing over the past 18 months (see 'Victims of violence'). More than 2,000 scientists are thought to have fled abroad.

Lack of investigation and prosecutions means little is known about the motives of the killers. Dlawer Ala'Aldeen, an Iraqi microbiologist at the University of Nottingham, UK, cautions that it is generally impossible to attribute assassinations to any one cause or group.

Some academics were part of the apparatus of Saddam Hussein's Ba'ath party and were victims of revenge, particularly in the aftermath

of Hussein's fall, says Ala'Aldeen.

But he believes there is now a broader range of political drivers. "The universities reflect the power struggles among the various groups in wider society," he says, with a war being waged between secular, Islamic and other factions. "It's so easy these days to lose your life in Iraq."

The breakdown in security and stability under the US occupation has also led to spiralling levels of organized crime and corruption. Mohamed Al-Rubeai, an Iraqi chemical engineer at University College Dublin, Ireland, thinks academics are killed simply as part of wider attempts by "terrorists and Ba'athists" to target anyone trying to restore normality in Iraqi society.

Nonetheless, many scientists — including Ala'Aldeen — are convinced that academics are being singled out. "Some of these murders are instigated by greed and criminality," says Rafid Alkhaddar, a water engineer at Liverpool John Moores University, UK. "But I and a vast number of Iraqis believe that there is an organized campaign to eliminate any remaining intellectuals inside Iraq."

"Terrorist forces are out to scare the scien-

tific community," agrees Abbas Al-Hussaini, a civil engineer at the University of Westminster, UK, and general-secretary of the Iraq Higher Education Organizing Committee (IHEOC) in London, created in January 2004 to help reconstruct Iraq's devastated research and higher-education system.

Scientists and academics in Iraq enjoy "much greater prestige and status than in the West, and could transform it into a modern society," says Al-Hussaini. "That is why they are being targeted." He too believes the main perpetrators are former members of the Ba'ath party.

Some Ba'athists are also cynically highlighting the plight of academics "to imply that the situation is worse than under Saddam", he claims. "In one way it is, but under Saddam's brutal dictatorship people had no rights; the future is now more hopeful."

Others take conspiracy theories further. The BRussels Tribunal, a Brussels-based people's court modelled on the Russell Tribunal, a US movement opposed to the Vietnam War, believes the killings are due to militia death squads associated with US forces. The tribunal is made up of prominent intellectuals, human-rights campaigners and non-governmental organizations, including linguist and left-wing campaigner Noam Chomsky, and Denis Halliday, former United Nations humanitarian coordinator in Iraq.

The tribunal launched a petition in April,



UNDER THE GUN

Hard figures on attacks in Iraq are hard to come by. Estimates for the number of attacks on academics between April 2003 and May 2006 vary from around 250 to more than 1,000. Ismail Jalili, an ophthalmic surgeon based in London, believes the figure is around 550. Out of 307 reports that he analysed, he found the following:

74% of attacks were fatal

80% of attacks targeted university staff

62% of victims were educated to PhD level

31% of victims were scientists, **23%** medics

57% of attacks occurred in Baghdad, **14%** in Basra, and **11%** in Mosul

D.B.



Military forces are a common sight at Baghdad University's campus, pictured here and below left.

signed by many intellectuals including several Nobel prizewinners in literature, describing the killing of academics as the "decimation of the secular middle class — which has refused to be co-opted by the US occupation".

Ophthalmic surgeon Ismail Jalili, former president of the UK Iraqi Medical Association and a member of the board of the British Arab Medical Association, takes a similar line. Jalili, who fled Iraq in 1969 after being imprisoned and tortured, says he believes many of the killings bear the "hallmark" of professionals (see 'Under the gun').

But despite varying views about who is behind the killings, Iraqis agree that the assassinations are unlikely to stop soon, and that the targets need protection. Most killings take place on the way to or from work, so Mosa Al-Mosawe, president of Baghdad University, suggests building a residential complex on campus, with armed guards, to protect staff and their families.

Foreign universities need to shelter Iraqi researchers who have received death threats, says Al-Hussaini. Since the fall of Baghdad, Al-Aldeen has arranged for many Iraqis to work at the University of Nottingham. But

many Western institutions are unaware of the issue, says Al-Hussaini. "Often it is only a matter of getting researchers and their families out for a few months," he says, to get them out of the line of fire.

The insurgency has undermined university reconstruction efforts "beyond belief", says Al-Aldeen. For the first time, this year no Iraqi students or staff are coming to Nottingham. "They don't reply to e-mails; their focus has gone; they can't plan; their lives are shattered."

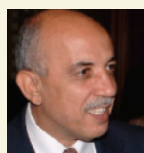
"Under mayhem and terrorism, a very advanced academic system, the star of the Middle East, has been reduced to nothing," he says. "It's heartbreaking to see a bunch of bright academics who should be rebuilding unable to do so."

One glimmer of hope is Kurdistan in the north of Iraq, where many of the country's scientists have moved. The region, which has been relatively autonomous from both Saddam Hussein and US forces, is a haven of relative security. University reconstruction is proceeding apace, and Al-Aldeen intends to hold the next IHEOC conference there next April. "It's symbolic; it will be in Iraq," he says. "We are excited about it."

D. VRANIC/AP

VICTIMS OF VIOLENCE

Ali Hassan Mahawish



Ali Hassan Mahawish, a materials researcher and dean of the engineering college at Mustansiriyah University in Baghdad, had been working on a project to develop unified design codes for the country's huge reconstruction programme. "It was vitally important for all engineering design and construction work in Iraq," says Abbas Al-Hussaini, a civil engineer at the University of Westminster, UK, who was Mahawish's partner on the project. Both men had also worked since 2003 on a scheme to twin researchers in Iraq with counterparts abroad.

In February, as Mahawish was being driven to work with his wife, armed men intercepted the car and asked his wife and driver to leave, recalls Al-Hussaini. "They phoned with a ransom demand, but a few days later his wife found his body dumped outside her door." Al-Hussaini is convinced that

Mahawish was killed because of his involvement in the reconstruction.

In an obituary on 12 March, the Arab Science and Technology Foundation mourned Mahawish as "a distinguished Iraqi scientist and sincere academic". He had been one of 20 top Iraqi scientists selected by the foundation to work on research "that addresses the needs and priorities of the Iraqi community".

Wissam Al-Hashimi



At the American Association of Petroleum Geologists annual meeting in Paris in September last year, renowned Iraqi geologist Wissam Al-Hashimi was due to give a paper on carbonate reservoirs in Mesopotamia. His slot was filled instead with a memorial service, recalls Muhammad Ibrahim, a geologist with Target Exploration in London.

Al-Hashimi, an expert in the porosity of dolomite, was kidnapped on 24 August 2005; his

car was intercepted on his way to work in Baghdad. Ibrahim, who grew up with him and is a friend of the family, says they paid a ransom. But Al-Hashimi's daughter found his body two weeks later in a Baghdad hospital, shot twice in the head. "She went from mortuary to mortuary looking every day," says Ibrahim. "His eyes had been pulled out during torture. I didn't know human beings could go so low."

Al-Hashimi, who published extensively on sedimentology and water resources in the Middle East, had a long academic career abroad before returning to Iraq in 1972 to head the mineralogy wing of the Geological Survey of Iraq. When he died, he was a consultant and member of the board of the Iraqi Drilling Company, and secretary general of the Arab Geologists Association.

Sabah al-Jaf

Sabah al-Jaf, a top official at the Iraqi education ministry, was gunned down in the Al-Karradah area of Baghdad on 22 May this year; one of his bodyguards was also killed, and

a second seriously wounded. Koichiro Matsuura, director-general of the United Nations Educational, Social and Cultural Organization (UNESCO), condemned the assassination, and the escalating attacks on academics. "By targeting educators, the perpetrators of such violence are undermining the reconstruction of Iraq and jeopardizing the future of the country and of democracy."

Al-Jaf was the third senior ministry official to be assassinated. Last year, he introduced a system to put all exam results on the Internet, with the idea that greater transparency would reduce nepotism; in the past, students with connections in government could expect better results. UNESCO had several projects with al-Jaf and other ministry officials. "Education is one of the most vital sectors for the Iraqi people," says Mohamed Djelid, director of the organization's Iraq office in Amman, Jordan. "We are just trying to maintain a minimum, trying to keep schools and universities open and have exams held."

D.B.

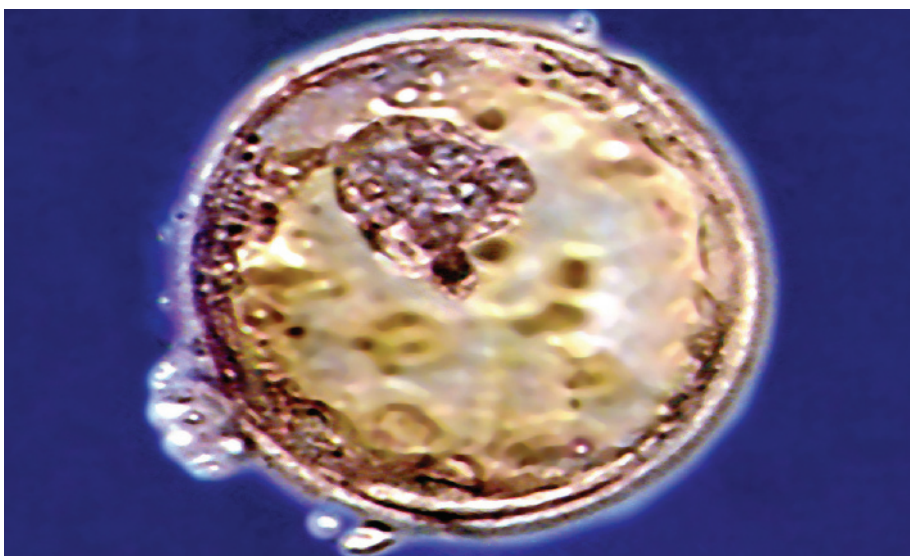
Human eggs supply 'ethical' stem cells

Human embryonic stem-cell lines have been successfully produced without using fertilized eggs. The researchers responsible work in Italy, which has some of the most restrictive embryo-research laws in the world. They hope the work will be welcomed as an ethically acceptable source of stem cells, as it does not involve destroying a viable embryo.

Embryonic stem cells are of great interest to medical researchers because they can transform into many different cell types, such as muscle, skin or brain cells. But there are ethical worries about pursuing such research, because creating an embryonic stem-cell line involves destroying a human embryo. Many countries have either banned the process in humans, or placed restrictions on the work that can be done.

One solution is to extract stem cells from parthenotes, embryo-like structures that develop from eggs without the need for fertilization. Some animals, such as insects and sea urchins, can reproduce in this way. Mammalian parthenotes, by contrast, always die before implanting in the womb, but they survive long enough to be a potential source of stem cells. Cell lines have been derived in the past from mice and monkeys. Now Tiziana Brevini and Fulvio Gandolfi of the University of Milan have achieved the feat in humans. From 104 eggs, donated by women at a local fertility clinic, they were able to derive two cell lines.

Because a parthenote does not have the genetic ability to form an individual, Brevini and Gandolfi have spent the past two years characterizing the cells to make certain that they behave like normal embryonic stem-cell



PHOTOTAKE/ALAMY

Source material: destroying this early embryo to create a stem-cell line raises serious moral issues.

lines. "We wanted to be sure before saying something," notes Gandolfi. The researchers announced their results at the annual meeting of the European Society of Human Reproduction and Embryology in Prague on 21 June, alongside other teams also trying to develop alternative sources of embryonic stem cells (see 'Philosophical approach').

The work is attracting high praise from other researchers in the field. "This is exciting. It was very important to show this in humans," says Alan Trounson, a stem-cell researcher from Monash University in Melbourne, Australia. He notes that the cells seem slightly more difficult to handle than normal embry-

onic stem cells, but adds that it is hard to tell whether this is likely to be a general characteristic of parthenogenetic lines. "If we can learn to handle them better, this could well be an acceptable alternative to embryonic stem cells," he says. Brevini and Gandolfi plan to submit their work for publication shortly.

The researchers do not think work on parthenotes will replace conventional embryonic stem-cell research. But Brevini says she hopes it will open the field to researchers in countries with strong restrictions. In Italy, for example, the creation or destruction of human embryos for research is banned, and the United States does not allow federal money to be used for such work. Parthenote lines "may be acceptable to the US administration, so they will release funds to work on these kinds of cells", she says.

Brevini adds that she prefers to work with these lines: "I feel more comfortable dealing with a parthenote than an embryo."

One secret of the group's success seems to be its relatively plentiful supply of eggs. Italy bans the creation of more than three embryos at once during *in vitro* fertilization, all of which must then be implanted. More than three eggs are often extracted from women undergoing the treatment, so they can donate those left over to researchers. Unfortunately, the supply is starting to dry up as women choose to freeze their extra eggs. ■

Jo Marchant

Read the conference blog here

♦ <http://blogs.nature.com/ESHRE>

PHILOSOPHICAL APPROACH

Some scientists are attempting to create embryonic stem-cell lines by removing single cells from mouse embryos at the eight-cell stage. The embryo remains viable after the cell has been removed, but the procedure still raises ethical questions because the single cell itself might have been able to produce a whole organism.

Takumi Takeuchi of Cornell University, New York, is one of those working on this approach in mice. After seeing a presentation of his results last

year, a philosopher called Matthew Liao, based at Johns Hopkins University in Baltimore, Maryland, contacted him with what he thought might be a better idea. Liao suggested letting the embryo develop further, until it consists of around 100 cells, and then taking four or five of those. Cells removed at this stage would still be able to form many different types of tissue, but would not be able to form a new individual.

At the Prague meeting, Takeuchi revealed that he and colleagues have now derived

stem-cell lines with reasonably high efficiency from 100-cell mouse embryos. The downside is that the procedure is quite invasive, and the survival rate of the embryos is cut in half. Researchers hope to improve that, but the transfer to humans will always pose an ethical problem.

If either approach could be made to work, individuals conceived through *in vitro* fertilization could have a stem-cell line banked for use in later life. "It would be insurance for the baby," says Takeuchi. **J.M.**

Pluto's children — a goddess and a monster

Pluto's two recently discovered moons now have names — Nix and Hydra. Last week, the International Astronomical Union formally approved the names for the moons, which were discovered last year (H. A. Weaver *et al.* *Nature* 439, 943–945; 2006).

Nyx was the Greek goddess of the night and the mother of Charon, for whom Pluto's biggest moon is named. But the Egyptian spelling, Nix, has been chosen for the moon, to avoid confusion with an asteroid already dubbed Nyx. Hydra was the nine-headed monster slain by Hercules; that moon's name refers to Pluto's status as the ninth planet.

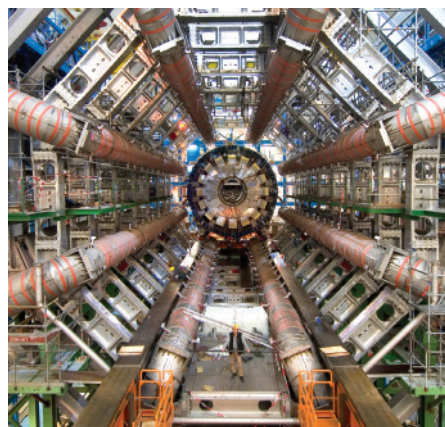
The initial letters N and H were also chosen as a nod to the New Horizons spacecraft, currently on its way to the planet. Pluto itself was named in part after the astronomer Percival Lowell, who began the search for the ninth planet.

Gentle start planned for European collider

Homeowners are often advised to allow builders twice the time they say they'll need. But the building of the world's most powerful particle-physics machine is coming in on schedule — almost.

The Large Hadron Collider at CERN, the European particle-physics laboratory near Geneva, had been scheduled to crash its first protons together in July 2007. That start date has been pushed back to November 2007.

The collider team has also decided to warm the machine up gently. Ultimately, particle beams will collide at an energy of 14 teraelectronvolts (TeV), but an initial two-month run will use beams colliding at just 0.9 TeV. Physicists will have to wait until April 2008, when the machine will start running at full energy, for any chance of glimpsing the new particles that they hope the collider will reveal.



Collisions on course: CERN's latest project is running just a few months behind schedule.

Raiders of the lost biodiversity

Renowned ecologist Edward O. Wilson (pictured left) now has his own foundation, formed by scientists, businessmen and an actor.

The E. O. Wilson Biodiversity Foundation launched on 23 June, with around 200 supporters engaging in a two-day 'BioBlitz' to identify species in New York City's Central Park. The foundation seeks to preserve biodiversity in part by creating BioTrust — a multi-corporation project that would financially reward nations that used their biodiversity to create marketable products.

Now retired from Harvard University, where he became



known for seminal work on ants, Wilson has long fought for environmental awareness. The board of his foundation, which is based in San Diego, California, includes Nobel-winning biologist James

Watson and Harrison Ford. The foundation's president and board chairman is Jay Short, former founder and chief executive of the bioprospecting firm Diversa of San Diego.

D. MCFADD/GETTY IMAGES

Academies exhorted to address gender bias

The world's science academies should be among the first to correct the under-representation of women in science, says a report from the Amsterdam-based InterAcademy Council (IAC).

Women typically make up less than 5% of the membership in the world's leading academies, including the IAC itself and the Royal Society in London. Less than 7% of the members of the US National Academy of Sciences are women. The Philippines' academy, the National Academy of Science and Technology, does slightly better: it has gender parity on its council board and has had a female president.

The 20 June report urges academies to acquire "good management practices" by creating balanced committees to study gender issues. It also recommends enlarging membership and leadership nomination pools to include more women.

Pal promises billions to Gates health charity

The Bill and Melinda Gates Foundation, funded by the world's richest person, is getting a donation from the second richest.

Warren Buffett, an investment expert from Omaha, Nebraska, announced on 26 June that he would give the bulk of his shares in his company, Berkshire Hathaway, to the Gates foundation, among other charitable organizations. The gift would currently see roughly \$30 billion of his \$44 billion fortune going to the charity.

The foundation, which already has a \$30-billion endowment, puts about half of

its money into global health programmes, including research into malaria, tuberculosis and HIV/AIDS.

In a letter to the foundation, the 75-year-old Buffett, who is a friend of the Gates couple, said that he was impressed with how the founder of Microsoft and his wife had worked on "improving the lives of millions of fellow humans who have not been as lucky as the three of us".

Heavy water and a lighter fluid

Here's one to ask your colleagues in the bar: why is it so much easier to prise a whiskey-soaked glass from a tabletop than a water-drenched one? The answer, according to chemists working in Sweden, lies in the vacuum that is briefly created when the glass leaves the table.

David van der Spoel of the University of Uppsala and colleagues found that the glass's resistance to an upward movement is partly due to the surface tension created where the liquid on the table meets the vacuum that forms under the glass. Alcohol molecules present in the liquid lower its surface tension. The energy needed to lift a glass resting on hard liquor is about half that needed if it's water (D. van der Spoel, E. J. W. Wensink and A. C. Hoffmann *Langmuir* 22, 5666–5672; 2006).

Correction

In our News Feature 'Magical mantle tour' (*Nature* 440, 1108–1110; 2006), Shigeaki Ono was quoted as saying that the SPring-8 synchrotron facility requires researchers to post data on a communal computer and official beamline notebook. Ono now says this is not a SPring-8 requirement, but rather a voluntary act.

00:00
MTSAT 140° E

00:12

00:23

SCIENCE ON THE SOLSTICE

Every day, all over the planet and beyond it, scientists try to make sense of the world in which they live. Here we present a composite picture of just one day — 21 June 2006, the Northern summer solstice.

Cerro Paranal, Chile

00:12 UT Morten Andersen is at the control console for Yepun, the fourth telescope in the European Southern Observatory's Very Large Telescope array. He is taking a census of the smallest stars of the Galaxy's most massive young cluster, Westerlund 1.

20:12 (20 June) local time
24°38' S 70°24' W

Bintulu, Malaysia

00:23 UT Diana James Junau, a project officer with the Planted Forests Project at Putra Malaysia University's Bintulu campus on Borneo, measures a molar tooth from one of a series of pig skulls. The skulls were collected as part of a five-year research project into the population biology of the bearded pig (*Sus barbatus*), to determine whether traditional food sources are sustainable when forests are turned into patchworks of cleared land.

08:23 local time 3°10' N 113°02' E

Low Earth orbit

00:26 UT The Hubble Space Telescope begins a 1,344-second near-infrared exposure. Anton Koekemoer of the Space Telescope Science Institute in Maryland will combine these data with subsequent exposures of the same field, obtained throughout the day, as part of the Hubble Ultra Deep Field project. The aim is to find distant galaxies

that were the first sources of light in the primordial Universe.

Oarai, Japan

01:00 UT At a test facility that is part of the JOYO experimental fast reactor, Takafumi Aoyama, a nuclear engineer for the Japan Atomic Energy Agency, consults and calibrates a temperature monitor. The monitor, which works on the basis of thermal expansion, has been developed for use in the reactor's core subassembly.

10:00 local time 36°19' N 140°36' E

Hanoi, Vietnam

01:30 UT Nguyen Van Hanh, a PhD student, shows Bui Xuan Nguyen some egg cells that have been matured *in vitro* for 22 hours. Nguyen needs the cells for his attempt to clone the saola (*Pseudoryx nghetinhensis*), a species of ox that lives only on the Laos–Vietnam border and is one of the rarest mammals in the world. Some of the swamp-buffalo oocytes look good and have clear polar bodies. Nguyen starts to remove the nuclei from the buffalo egg cells while a colleague prepares some saola cells, readying them for fusion around noon.

08:30 local time 21°01' N 105°30' E

Bangalore, India

02:55 UT This little girl, weighing 3.14 kg, is born just before 8 a.m. local time in Manipal Hospital — the first baby for proud mother



and father Rhadhika and Rajesh Sinha, who were still thinking of a name for her when this photo was taken. Hers is one of the estimated 358,522 new human lives starting on 21 June 2006, more than double the number (155,000 or so) ending. The estimated world population stands at 6,523,642,761. Babies born in India can currently expect to live for 63.3 years, some years shy of the global life expectancy of 67.

07:55 local time 12°58' N 77°34' E

Newcastle, Australia

03:15 UT Anne Imenes at Australia's National Solar Energy Centre is testing one of the 200 mirrors intended for the 'solar concentrator'. The experimental array will concentrate 500 kW of solar thermal power on to a reactor 17 metres above the ground, heating it to more than 1,000 °C. The reactor will be installed in July.

13:15 local time 32°53' S 151°44' E

Strait of Johor

03:45 UT Juan Walford and his colleague B. Sivaloganathan of the National University of Singapore are

checking on some six-month-old seahorses, which they are rearing in a floating fish farm off the east coast of Singapore. Their study aims to use the seahorses as living indicators of the marine environment. This morning they find a lot of 'pregnant' males with embryos in their brood pouch, indicating that the juvenile males have successfully reached reproductive age, and that it would be feasible to restock the area.

11:45 local time 1°24' N 103°58' E

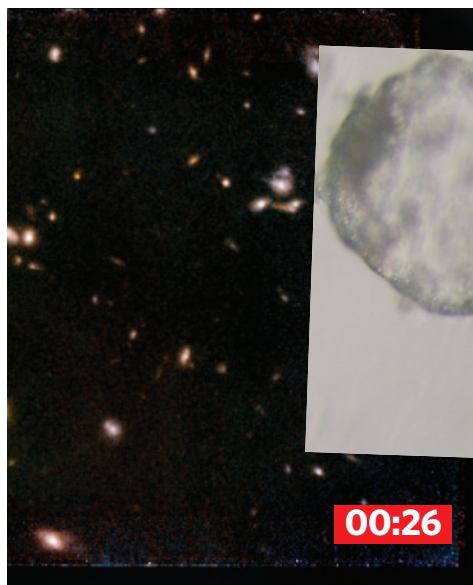
Florham Park, New Jersey

03:47 UT Dan Silver, marketing director for a multinational manufacturer of medical devices, e-mails ProMED-mail with a report that he has translated from a Chinese newspaper. The Internet site (www.promedmail.org) provides information on disease outbreaks and depends on doctors, researchers and health professionals around the world sending in information in this way. The report describes 60 students and teachers who have come down with an undiagnosed febrile illness in Shaanxi, China, since 12 June.

23:47 (20 June) local time
40°47' N 74°28' W

Tokyo, Japan

04:00 UT The hundreds of small mirrors, beam splitters and lenses in Akira Furusawa's quantum-computing lab at the University of Tokyo look like a mess; in fact they



01:30

00:26

are meticulously organized. One of his students is trying to beat the world record for enhancing the quantum correlation between photons.

13:00 local time 35°43' N 139°46' E

Krasnoyarsk, Russia

04:00 UT At the Sukachev Institute of Forest, Eugene Vaganov and Ernst-Detlef Schulze, visiting from the Max Planck Institute for Biogeochemistry in Jena, Germany, pore over a joint publication on carbon-isotope ratios in wood. They are trying to figure out how to disentangle the contributions of isotopes from photosynthesis, carbon storage, respiration and wood formation when interpreting the isotope patterns found in tree rings.

12:00 local time 56°00' N 92°47' E

New York, New York

04:33 UT In a dark, cramped lab at Columbia University Medical Center a microscope automatically snaps images of live yeast cells as they divide. Joanne Bruno is trying to find out which fluorescently tagged proteins behave asymmetrically during cell division, segregating unevenly between daughter cells.

00:33 local time 40°48' N 73°58' W

Tidbinbilla, Australia

04:35 UT At the Deep Space Network station outside Canberra, a 34-metre radio dish starts to communicate with the Mars Odyssey orbiter, 343.6 million kilometres away. Data will pass between the ground station and the satellite for the next 4 hours 35 minutes.

14:35 local time 35°26' S 148°56' E

Würzburg, Germany

05:11 UT At the wine laboratory of the Bavarian Health and Food Safety

Authority, Norbert Christoph is switching on the sample changer of the 400-MHz NMR spectrometer. The device will determine the deuterium:hydrogen isotope ratio of ethanol and sugar in two Macedonian wines and three apple juices. In less than an hour, the staff of the institute will leave for their annual outing — an excursion by bus to the city and castle of Langenburg. After a visit to the old town, the castle and a vintage car museum they will enjoy some local food and wine. Meanwhile, back in the lab, the NMR wine spectrometer is pulsing at 61.4 MHz every 7 seconds in order to measure the deuterated ethanol molecules.

07:11 local time 49°47' N 9°56' E

Munich, Germany

06:24 UT Martin Hrabé de Angeles enters the German Mouse Clinic at the National Research Centre for Environment and Health. Today he and his colleagues are going to start characterizing new mouse models for bone-related diseases. By the end of the day they will have made a total of 1,636 measurements of characteristics such as bone density, strength and biochemical composition. While gathering these data, they will discuss ways in which models may shed light on the molecular basis of bone diseases.

08:24 local time 48°07' N 11°34' E

Bethlehem, Palestinian National Authority

06:30 UT Moein Kanaan drops off some DNA sequencing reagents for his lab at Bethlehem University. Having an Israeli ID makes it easier for him than for many of his colleagues to move such reagents across the Israel–Palestine border. The next job of the day is to order a

new spectrophotometer called a Nanodrop, an instrument that measures minute amounts of DNA, RNA and protein. Kanaan is trying to squeeze the supplier of the Nanodrop for as big a discount as possible.

09:30 local time 31°42' N 35°12' E

Copenhagen, Denmark

06:37 UT Erik Born of the Greenland Institute of Natural Resources is writing to the Danish Ministry of Justice for a permit to export and re-import two crossbows and a tranquillizer gun that he wants to take to Canada. He hopes to use the equipment to take skin biopsies from walrus on Baffin Island in August and to attach satellite transmitters to some of the animals.

08:37 local time 55°41' N 12°36' E

Low Earth orbit

06:46 UT On board the International Space Station, Jeffrey Williams photographs a 19-km-diameter crater in the Australian outback that is more than 515 million years old. The snapshot, the first of three Williams will take today, is part of a series of Earth observations that the station does for a variety of different scientific projects.

Frederiksberg, Denmark

07:10 UT At a supermarket run by the Danish Department of Human Nutrition, six overweight families collect food and drink for the next couple of days. This is part of Diogenes, a six-month dietary-intervention trial sponsored by the European Union. All the products are supplied to the volunteers free of charge, and the bar codes on the food items are used to declare nutritional information, which is registered by a computer linked to

the barcode scanner at the checkout. Two families complain that the researchers are out of fresh chicken breasts.

09:10 local time 55°25' N 11°34' E

Tsukuba, Japan

07:24 UT At the KEK B factory, birds are singing despite the overcast day. The electron and positron beams in the accelerator are colliding with a brightness that far outshines any other accelerator facility. The factory's Belle experiment has so far recorded more than 500 million pairs of B mesons. Like many at the facility, Jasna Dragic is analysing the large data sample and preparing a talk on the latest results, which she will present at the International Conference on High Energy Physics in Moscow. Her colleague Ruslan Chistov is impatiently waiting for final approval from Belle spokespersons to submit a manuscript to *Physical Review Letters* reporting the discovery of two new particles.

16:24 local time 36°09' N 140°04' E

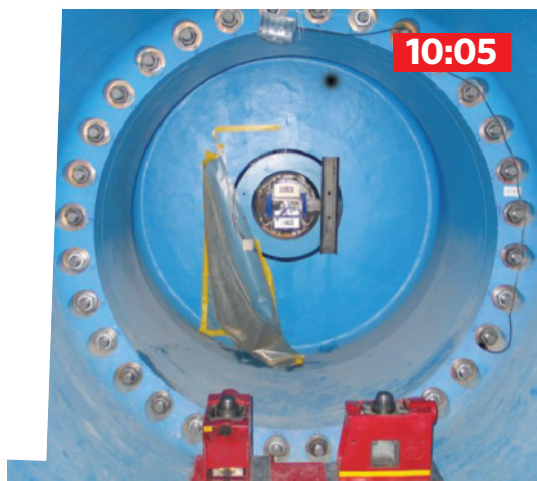
Prague, Czech Republic

07:27 UT Catherine Staessen of the Flemish-speaking Free University of Brussels has just made her presentation at the annual conference of the European Society of Human Reproduction and Embryology. She has analysed the limited trial data available so far on the value of screening embryos created by *in vitro* fertilization for chromosomal abnormalities before they are implanted. She concludes that there is no evidence that screening increases a woman's chance of having a healthy baby. "You have a beautiful approach but the wrong answers!" complains Yuri Verlinsky, director of the



03:45

10:02



Reproductive Genetics Institute in Chicago and a pioneer of preimplantation genetics. "Abnormal human embryos are an obvious fact, so if you remove those, how can there be no benefit?" Staessen's response: do the trials and prove to me it works.

09:27 local time 50°04' N 14°26' E

The Alexander/Zomar stream, Israel

08:05 UT Lior Asaf of the Arava Institute for Environmental Studies in Israel and his Palestinian partner Nader Al Khateeb of the Water and Environmental Development Organization in Bethlehem are measuring the flow of a stream using an electromagnetic velocity meter. The work is a part of a project to estimate the pollution from different sources that ends up in the Alexander/Zomar stream, which crosses the boundaries between Israeli and Palestinian lands.

11:05 local time 32°21' N 34°57' E

Kunming, China

08:35 UT Weizhi Ji is preparing to view several rhesus-monkey embryos and cloned blastocysts obtained by *in vitro* fertilization under a confocal microscope at Kunming Primate Research Center. He is interested in comparing indicators of gene activation in the cells that go on to form the embryo itself and those that form the placenta and amniotic membranes.

16:35 local time 25°04' N 102°42' E

Peradeniya, Sri Lanka

08:40 UT Kapila Dahanayake, a geologist at the University of Peradeniya, is analysing a sample of water and sediment from the tsunami of December 2004 that was preserved in an empty *arak* bottle

found in a flooded house. The sediment contains microplankton typical of the deep ocean and has a particular distribution of grain sizes. They are very like the sediments found at a depth of 1.5 metres in a nearby well, which supports the idea that those sediments too were laid down by a tsunami.

14:10 local time 7°15' N 80°34' E

Bellville, South Africa

08:45 UT At the South African National Bioinformatics Institute, Winston Hide analyses a set of mutations in HIV that have resulted from treatment with the antiretroviral drug nevirapine, used to prevent transmission of the virus from mother to child. He then leaves for the airport to pick up his collaborator Harukazu Suzuki, who has spent 21 hours getting to South Africa from the RIKEN Genomic Sciences Center in Yokohama, Japan. On the way, he drives through shacklands and past HIV-awareness posters, and jockeys for space with horse-drawn carts.

10:45 local time 33°56' S 18°38' E

Robledo de Chavela, Spain

08:55 UT As Mars sinks in Australia's western sky, it rises in the east for Spain. A Deep Space Network 34-metre dish near Madrid, almost identical to the one outside Canberra, takes up the job of bringing home data from Mars Odyssey. This includes data relayed through the satellite from the Spirit rover, currently encamped for the winter on a north-facing slope in the Columbia Hills of Gusev Crater.

10:55 local time 40°30' S 4°14' W

In transit, Frankfurt to Manila

09:00 UT In a plane somewhere over the Indian Ocean, the chairman

of the Intergovernmental Panel on Climate Change, R. K. Pachauri, is on his way to a meeting of the Asian Development Bank. He picks up an e-mail from the vice-president responsible for sustainable development at the largest retailing organization in the world. The two men met less than a week ago in Iceland. The message includes an invitation to a meeting next month where 150 senior corporate leaders will discuss climate change.

Mauna Loa, Hawaii

09:00 UT The average concentration of carbon dioxide in Hawaii's air on 20 June has just been calculated: 383.63 parts per million. The data from Mauna Loa constitute the longest record of direct measurements of CO₂ in the atmosphere.

23:00 (20 June) local time 19°32' N 155°35' W

Ambohimiravavy, Madagascar

09:23 UT At the summit of Ambohimiravavy (2,310 m), Martin Callmender and his team from the Missouri Botanical Garden in St Louis are collecting plants on the unexplored and hard-to-reach massif.

12:23 local time 14°12' S 49°6' E

Western Pacific Ocean

09:50 UT A measuring device that has been moored to the bottom of the ocean bobs to the surface, ready to be picked up by Randolph Watts and Kathleen Donohue, from the University of Rhode Island, and their team on the *Melville*, a ship operated by the Scripps Institution of Oceanography. The surfacing instrument (a 'current and pressure recording inverted echo sounder', or CPIES, pronounced 'sea-pies') contains two years of data on the

conditions in this cold mixed-water region. Picking up these devices is usually straightforward. This time, however, the surfacing CPIES has inadvertently turned off its relocation radio and strobe-light, and the sea is covered in pea-soup fog. It takes five hours to find it and haul it safely aboard.

23:50 ship's time 39°31' N 148°20' E

Alexandria, Egypt

10:02 UT School students from Alexandria gather in the plaza of the new Bibliotheca Alexandrina to measure Earth's circumference using the method that Eratosthenes pioneered nearly 2,000 years ago. Eratosthenes — the third director of the original library of Alexandria — observed that, on the solstice, the Sun's reflection could be seen at the bottom of a well on Elephantine Island, near Aswan, and must thus be directly overhead. By measuring the length of a shadow on the same day near Alexandria, he was able to calculate Earth's radius geometrically. Today, children in Alexandria are making one measurement while children near Eratosthenes' well in Aswan make the other; they compare results in a videoconference. "We did it! We measured Earth's circumference!" the Alexandrians shout out when the calculations come good.

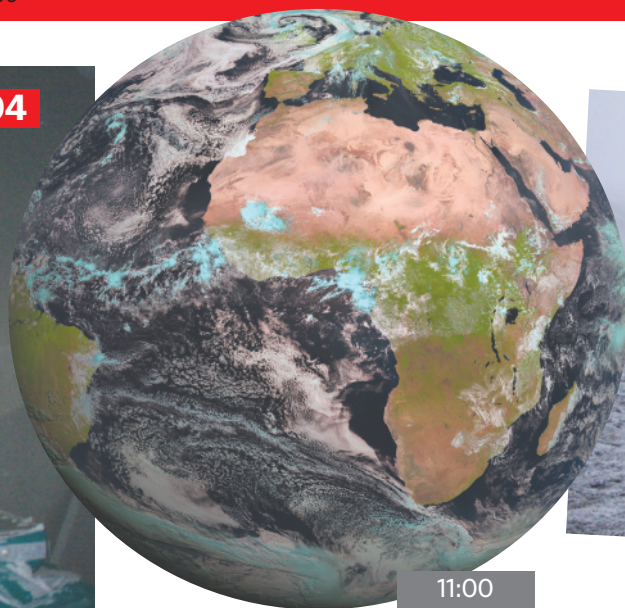
13:02 local time 31°13' N 29°57' E

CERN, the border of Switzerland and France

10:05 UT Having spent much of the morning finishing a paper for next week's European Particle Accelerator Conference in Edinburgh, Mike Lamont takes the lift down to the 27-km tunnel of the Large Hadron Collider. Pasted on its wall, the lift has a large schedule for football's



10:04



11:00

Meteosat 0°



15:22

World Cup; graffiti indicate, surprisingly, that it is about to be won by Morocco. In the evening, a beer outside a bar in St Genis beckons — but so does work on a second paper for the Edinburgh meeting.

12:05 local time 46°14' N 6°03' E

Amundsen-Scott station, Antarctica

10:54 UT After his daily 20-minute walk across the frozen polar plateau from the new elevated South Pole station to the Dark Sector Lab, Denis Barkats transfers 90 litres of liquid helium and 40 litres of liquid nitrogen into the cryostat for the BICEP telescope (for Background Imaging of Cosmic Extragalactic Polarization). The telescope makes use of the long, cold Antarctic nights to gather up as many photons as possible from the earliest days of the Universe. The liquids keep the array of 98 polarization-sensitive bolometers at the telescope's heart at a temperature of 250 millikelvin. The outside world isn't quite that cold — just -65 °C. And the atmosphere in the station is positively festive as the 64 'winterovers' celebrate midwinter night. In three months exactly, the Sun will rise again.

22:54 local time 90° S 0°

Culham, UK

10:55 UT Dragoslav Ciric coaxes the first pulses of deuterium through the Culham Science Centre's new neutral beam injector, which will eventually be used to heat plasmas in the Mega Amp Spherical Tokamak (MAST), a nuclear-fusion experiment run by the UK Atomic Energy Authority. Until now, MAST has relied on two injectors on loan from the Oak Ridge National Laboratory, but these use

1960s technology and deliver only short beam pulses of just a quarter of a second. By mid-July, researchers should be ready to investigate the effect of high-power, long-pulse injection on MAST plasmas.

11:55 local time 51°39' N 1°14' E

Ny-Ålesund, Svalbard

11:05 UT Ruth Müller, Franciska Steinhoff and Max Schwanitz, researchers at the Franco-German AWIPEV base, head out to sea in a zodiac to measure light intensity in the water. AWIPEV's scientists work on all aspects of the environment, releasing balloons to measure ozone levels in the stratosphere and diving into the sea to pull up sediment samples. The light-intensity measurements are part of a project to assess the influence of ultraviolet radiation on algae in the Arctic Ocean.

13:05 local time 77°34' N 23°42' E

Bangalore, India

11:25 UT Myslswamy Annadurai, project director for Chandrayaan-1, India's first mission to the Moon, sits down with two of his engineers for a final check of the spacecraft simulator systems they will be taking to the United States in two days. There they will be used to check the interfaces for two US instruments that will be included in the mission. The spacecraft is due for launch next year.

16:55 local time 12°58' N 77°34' E

Borneo, Indonesia

12:00 UT Chairul Nidom, a virologist at Airlangga University's tropical-disease centre in Surabaya, Java, visits the Orangutan Rehabilitation Center in Palangkaraya to collect blood and trachea swab samples from

orangutans. The trachea swabs will be tested for influenza viruses, including bird flu, and tuberculosis. The blood sera will be analysed for hepatitis viruses. It's the end of a busy day for Nidom, who began by giving an 8 a.m. lecture to veterinary students in Java on animal welfare, followed by an afternoon lecture to vets in Palangkaraya on the H5N1 bird-flu virus in chickens, pigs, wild birds and humans.

20:00 local time 2°16' S 113°55' E

Nouragues Station, French Guiana

12:10 UT Following the station's temporary evacuation after two murders by illegal gold miners last month, work is beginning again under the permanent protection of the gendarmes. The tapir team is recovering photos from the 22 trap cameras set in 9 square kilometres of forest. The ONCFS, France's office for hunting and wild fauna, wants to compare the tapir densities in regions where they are hunted, and in the undisturbed region of Nouragues.

09:10 local time 4°05' N 52°40' W

Oxford, UK

12:25 UT Overheard at lunch in a neuroscience lab: "They've only had 27 applications and they're supposed to fund ten of them. I really like those odds."

13:25 local time 51°45' N 1°15' W

Worldwide

12:26 UT At the moment of the solstice, the Sun is as high as it gets above the plane of the Equator.

Ewa Beach, Hawaii

12:35 UT Gerard Fryer is fast asleep in the trailer behind the Pacific Tsunami Warning Center when his

pager starts bleating. Twelve thousand kilometres away an earthquake has been picked up by seismometers. He pulls on his clothes and runs to the operations building to discover that Dailin Wang, the scientist on watch, has already located and measured the earthquake: it was in the Nicobar Islands and of magnitude 5.9. This is not large enough to create a tsunami, so they send a message saying so.

02:35 local time 21°19' N 158°01' W

Rockville, Maryland

13:05 UT Jeffery Taubenberger is packing up his office and lab at the Armed Forces Institute of Pathology ready for his move to the National Institutes of Health, where he will set up a research programme on flu pathogenesis. He is also in the process of analysing initial sequence fragments from pre-1918 human influenza cases obtained by screening autopsy lung tissue samples for influenza A with a DNA amplification protocol. This should allow him and his colleagues to determine in the long run whether any parts of the pre-1918 human flu virus were carried forward into the pandemic virus of 1918.

09:05 local time 38°58' N 77°02' W

Worldwide

14:00 UT The Interacademy Panel on International Issues, representing the world's national science academies, releases a joint statement on the teaching of evolution. The statement urges parents and teachers to provide children with the facts about the origins and evolution of life on Earth.

Manhica, Mozambique

14:05 UT After getting back from the World Health Organization



15:30

office in Maputo where he was planning a meeting on the introduction of possible malaria vaccines in Africa next year, Betuel Sigauque, a doctor and public-health worker, is catching up on e-mails when he's interrupted by bad news. The generator in Tãninga, one of the outlying areas involved in Sigauque's malaria research, is misbehaving, and someone will have to go out there and bring research materials back to the main lab in Manhica.

16:05 local time 25°24' S 32°48' E

Atlantic Ocean

14:35 UT A float that spends most of its life drifting well below the water pops to the surface off the northwest of Ireland and transmits a burst of data to a satellite. It is one of 2,447 floats in the Argo system, which travel the world mapping out currents and measuring water temperatures. The satellite sends the data down to a ground station outside Toulouse, France. An



16:43



16:59

automatic program at the British Oceanographic Data Centre in Liverpool pulls the data across, checks the quality, reformats and passes them on to one of the three international Global Data Assembly Centres, where they are merged with data from all the other nations that contribute to the Argo programme.

15:35 local time 56°12' N 11°15' W

Pika Camp, Canada

15:22 UT As the clouds lift from the mountain peaks of the Yukon, Sarah Trefry sets off to a nearby boulder field to record the vocalizations of collared pikas (*Ochotona collaris*). Her work is part of a study into the effects of rapid climate warming on the dynamics of alpine ecosystems. Although the past winter was one of the warmest on record, the late spring snowdrifts are melting slowly and the alpine meadows are only just beginning to show the first signs of summer life.

08:22 local time 61°08' N 138°10' W

South Atlantic Ocean

15:30 UT The *Polarstern* is on her way south to the Southern Ocean to perform a study of overwintering mechanisms of krill and other organisms. The crew and scientists are using the few daylight hours to prepare the plankton nets. This is not so easy in a force 8.0 and with six-metre waves.

15:30 ship's time
46°29' S 07°31' E

Stockholm, Sweden

15:45 UT Tomas Nyman of the Structural Genomics

Consortium (SGC) lab at the Karolinska Institute is enjoying his afternoon coffee, eating fresh strawberries and cream on the sunny balcony outside the lab. The working day in Stockholm will soon be over, although the sun will continue to shine for a good five hours. The other two SGC laboratories, in Oxford and Toronto, will continue to operate into the Swedish night. By the time the Canadians end their day, the SGC will have deposited structures for ten human proteins in the Protein Data Bank.

17:45 local time 59°17' N 18°04' E

Atlanta, Georgia

16:20 UT At the Centers for Disease Control and Prevention, Fred Tenover is dismayed by the outcome of his latest study. His team has been looking at the automated systems that identify antimicrobial-resistant bacteria. Tenover has just found that several of the systems fail to identify the strains of multidrug-resistant *Staphylococcus aureus* and *Enterococcus faecium* that are known to be resistant to linezolid. If labs can't detect these strains, they will tell doctors that a patient's infection should respond to the drug, when it might not. This is not the 20th wedding anniversary present that Tenover was anticipating.

12:20 local time 33°43' N 84°23' W

Sutherland, South Africa

16:42 UT At dusk on the high plateau of the Great Karoo Desert, staff astronomers Encarni Romero Colmenero, Nicola Loaring and Fred Marang carefully align the 91 individual segments of the South African Large Telescope's 11-metre mirror. Tonight's scientific programme has been designed to test the instruments and subsystems

of this fledgling observatory. They will be looking at a gravitationally lensed quasar, black-hole candidates in the Galactic bulge, dynamic winds from Wolf-Rayet stars and giant radio galaxies.

18:42 local time 32°23' S 20°49' E

New York, New York

16:43 UT The American Museum of Natural History has invited the press 'behind the scenes' to preview a snakes and lizards exhibition. Postdoc Jack Conrad is showing off a slab of orange sandstone collected from the Gobi Desert that contains the delicate, 84-million-year-old skeleton of a recently discovered lizard species. The grey-banded kingsnake vanishing up curator Darrel Frost's sleeve, meanwhile, is neither an exhibit nor a piece of research; it's a pet.

12:43 local time 40°47' N 73°58' W

Yellowstone National Park, Wyoming

16:59 UT In Amphitheater Springs, Tim McDermott and graduate student Dana Skorupa remove samples of algae from water that can reach 72 °C. The researchers, based at the Thermal Biology Institute of Montana State University, will extract messenger RNA from the algae for use in microarrays. They are studying which genes get turned on or off in June, when levels of visible and ultraviolet light reach a peak, and the algae die off in a big way.

10:59 local time 44°48' N 110°44' W

Ambo, Peru

17:00 UT An international team of scientists sponsored by the National Geographic Society is deep inside a cave, partway up a cliff that overlooks Ambo — some 2,000 metres up in the Andes. Bruce

17:45
GOES EAST 75° W



Shockey of the American Museum of Natural History is excavating a partial skeleton of a large sabre-toothed cat; 20 metres below him, Rodolfo Salas of Lima's Natural History Museum discovers the jaw of a new sloth. French colleagues map the labyrinth known locally as Jatun Uchco, the 'profound hole'. Later, on his way to an Internet café, Shockey gets "caveman dust" all over the people sharing his taxi.

12:00 local time 10°08' S 76°12' W

Muscat, Oman

17:03 UT In her office at Sultan Qaboos University, botanist Annette Patzelt finishes formatting the draft manuscript of the Oman Plant Red Data List. The book contains detailed information about the conservation status of 262 Omani plants; it is the first such register in the whole of the Arabian peninsula.

21:03 local time 23°36' N 58°32' E

Waterloo, Canada

17:15 UT "How do you make a clock out of light?" asks Olaf Dreyer, a visitor having lunch at the Perimeter Institute for Theoretical Physics. He wants to figure out whether it is possible to use light beams to define time in a theory of quantum gravity. Fotini Markopoulou and Daniel Gottesman from the institute kick the idea around with him for a bit before more people arrive and the conversation inevitably turns to the World Cup. No practical plans for a light-clock emerge.

13:15 local time 43°28' N 80°32' W

Worcester, Massachusetts

17:54 UT Daniel Rowe and his mentor Douglas Golenbock raise a glass to celebrate Rowe's successful defence of his PhD thesis. Rowe

identified a protein that interacts with an immune-system molecule called toll-like receptor 4, or TLR4. The work took him five years; he has yet to decide his postdoc position.

13:54 local time 42°16' N 71°48' W

Moorea Island, French Polynesia

18:29 UT Gustav Paulay and Sally Holbrook of the Gump Research Station collect coral symbionts from a reef just after dawn. Paulay is part of the Moorea Biocode Project, which aims to catalogue the entire flora and fauna of this volcanic island. Holbrook studies the complex system of coral reefs and lagoons that surround the island. The animals in the early-morning catch will be identified, photographed and genetically barcoded for use in studies of the reef's food webs and populations.

08:29 local time 17°30' S 149°50' W

Buenos Aires, Argentina

19:00 UT Everyone in the organic-chemistry department of Buenos Aires University is gathered in front of the department lounge's television to watch the Argentina–Netherlands football match. All research activity has stopped except for the NMR spectrometer next door, which is measuring a carbon-13 spectrum — something it will manage to do uninterrupted for the next 90 minutes.

16:00 local time 34°12' S 58°18' W

Gröningen, the Netherlands

19:00 UT The staff, students and researchers from Ben Feringa's synthetic organic chemistry lab at Gröningen University, having enjoyed their annual barbecue at the boss's house, are gathered indoors in

front of a big screen for the Netherlands–Argentina match. Even those who come from neither country are pretty excited.

21:00 local time 53°13' N 6°34' E

Pasadena, California

19:04 UT At the Jet Propulsion Laboratory, Jon Giorgini updates the orbit of asteroid 2004 XP14 and sends the tracking data to the Goldstone planetary radar facility in the Mojave Desert. 2004 XP14 will come almost as close to Earth as the Moon is on 3 July; it will be the closest asteroid approach ever observed by radar.

12:04 local time 34°12' N 118°11' W

Denver, Colorado

19:12 UT Bruce Williams, who teaches 13-year olds at Joyce Kilmer School in Trenton, New Jersey, is registering a team to compete in the National Middle School Science Bowl in Denver. His students, Roger Barrett, Niyi Odumosu, Dymond Ruffin and Dahlia Wesley, will compete in a hydrogen fuel-cell model-car race: the fastest over 10 metres wins.

13:12 local time 39°41' N 104°58' W

Norman, Oklahoma

20:00 UT Samuel Mbainayel, a meteorologist in Chad's civil service, takes his wife and daughter to the local health centre for immunizations. When he gets back, he returns to the literature review he is doing for a paper on how variability in rainfall in the Soudano-Sahelian zone of west Africa might be affected by interactions between land surface and the atmosphere, which will form part of the master's thesis he's over in the United States to work on.

15:00 local time 35°13' N 97°26' W

Ondřejov, Czech Republic

20:45 UT It's not a good night for the automated observatories in the Czech Republic as they scan the skies for fireballs — haze and clouds are affecting the view. The only trails observed are caused by planes and the International Space Station. Instead, Pavel Sporný spends the evening analysing the trajectory of a meteor spotted over the remote Nullarbor Plain in southwest Australia on 8 March, captured on disk by collaborators at the Western Australian Museum in Perth.

22:45 local time 49°54' N 14°46' E

Bar Harbor, Maine

21:35 UT The Jackson Laboratory freezes its 3,052,986th mouse embryo. There are now 2,612 strains of mice cryopreserved in the labs' vaults. Also passing through the cryopreservation service today: 1 fresh sperm order, 1,597 two-cell embryos frozen in 38 straws, 40 embryo transfers and 3,084 oocytes. One order was shipped.

17:35 local time 44°22' N 68°12' W

Malargüe, Argentina

23:04 UT At the Pierre Auger Observatory, after hours of careful calibration, researchers begin to take their data. Eighteen telescopes spread across the *Pampa amarilla* of western Argentina begin the hunt for the glow of cosmic rays from far beyond the Galaxy. The wind is a little high, but the skies are clear — and the night will be the longest that the team will have all year.

20:04 local time 35°28' S 69°35' W

There is a greatly expanded version of this feature online

► www.nature.com/news/specials/solstice

BUSINESS

Assault on batteries

Miniature fuel cells are being developed as an alternative way to power portable devices. But they're not ready yet, as Kurt Kleiner reports.

For years manufacturers have flirted with the idea of using fuel cells to drive portable electronic equipment. Their engineers envisage power sources that can, with just a few cubic centimetres of fuel, run a laptop all day — or a phone for a week.

The most recent step forward came in May, when Samsung announced that it would invest in MTI Micro, a company based in Albany, New York. The South Korean firm wants MTI to help it develop a fuel cell for a mobile phone, and it's not alone. Toshiba, Sanyo, Panasonic and others have all been researching fuel cells for some time. But it is not yet apparent when these fuel cells will come to market — or even whether they will make a real impact.

"I think there's enough hype that everyone wants to make sure they don't miss the bandwagon. But it doesn't seem that the big electronics companies are going into it full force," says Jeremiah Bryant, a senior research analyst with Darnell Group in California.

Advocates say that small fuel cells would provide three to ten times as much power as even the best battery, allowing people to run their laptops all day without recharging, or to watch entire movies on their mobile phones. But fuel cells have technical and financial hurdles to overcome before they can hope for a significant portion of the US\$7-billion portable-battery market.

Reaching the limit

"Batteries are great," admits Ged McLean, president of fuel-cell maker Angstrom of North Vancouver, British Columbia. "They're a formidable technology." And yet that technology may be hitting its performance limits, according to McLean and industry analysts. Every year batteries manage to eke out about a 5% gain in performance. But there is no technology on the horizon that is set to dramatically increase this, says Sara Bradford, a battery-industry analyst for Frost and Sullivan in Dallas, Texas.

Fuel cells could, in theory, blast through the battery barrier. Like a conventional battery, a fuel cell uses a chemical reaction to generate an electric current. However, in a conventional battery, once the reaction is exhausted the battery is either thrown away or electrically recharged. With a fuel cell, you simply

add more of the fuel that has been used up.

There are several potential advantages to fuel cells. You don't have to wait for a recharge. Performance does not degrade over time as with rechargeable batteries. Best of all, the chemicals used in a fuel cell are much more energy dense than those in batteries, so you get a lot more electricity for a given weight of chemical.

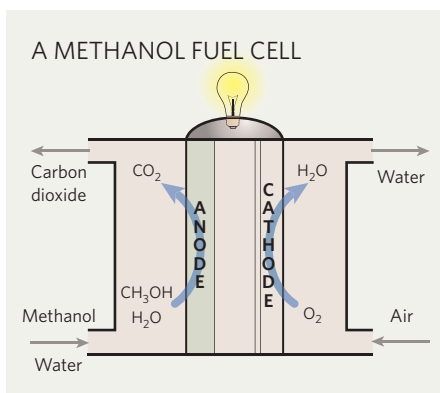
For instance, a lithium-ion battery will deliver 150 watt-hours per kilogram, whereas a fuel cell could deliver up to 1,200 watt-hours per kilogram, according to the company Smart Fuel Cell of Brunthal, Germany.

Most fuel cells consume hydrogen and produce water, but issues with storage of the potentially explosive gas may preclude its use in portable devices. Angstrom has tackled this by using solid metal hydrides — metal crystals that bind hydrogen to their surfaces. When the fuel cell runs low, the metal hydrides can be refilled with hydrogen from an outside source.

Most companies are simply trying to use another fuel: methanol. This has the advantage of being cheap, and easy to store because it is liquid at room temperature. Consumers would buy specially made methanol cartridges and click them into the fuel cell when needed. In theory, these cartridges would be as safe, cheap and ubiquitous as disposable cigarette lighters.

The methanol fuel-cell industry won an important victory late last year when the International Civil Aviation Organization voted to allow airline passengers to carry methanol fuel cells on aircraft, starting in January 2007. Previously, methanol had been banned as a hazardous material.

But the methanol fuel cell has potential



problems, because the chemistry isn't quite as straightforward as that of a hydrogen cell. A direct methanol fuel cell uses water during the first half of the chemical reaction, and produces water in the second half (see graphic). Designers therefore have to circulate water from the cathode back to the anode.

Caravan power

Smart Fuel Cell has already had some success with methanol. The company makes a standard 12-kg methanol fuel cell for recreational vehicles. The key now is to produce a small cell that is as dependable and efficient as the larger ones, says Jens Müller, the firm's managing director.

The task is complicated by the need for internal pumps to move water and fuel, as well as fans and filters to provide fresh air. These add weight and complexity to the cell. But Müller says that's a solvable engineering problem.

"This technology isn't as complex as it seems. We're using components that are not fuel-cell specific. They're used in other industries, and they're rugged," Müller says.

MTI, which is receiving a small initial investment of \$1 million from Samsung, is trying a different tack. It wants to build a 'passive' methanol fuel cell, perhaps using absorption and diffusion to move water and fuel around. Although the cell would still have some moving parts, MTI thinks the passive approach will be cheaper and easier to implement.

MTI, Angstrom, Smart Fuel Cell and others



REUTERS/Y. NAKAO

Talk it up: fuel-cell developers hope to power phones with tiny methanol plug-ins.

are trying to find initial markets in industry and the military, in applications where long run times are so important that the customer will pay a hefty premium. In May, Smart Fuel Cell announced a \$500,000 contract with the US Air Force to provide a prototype fuel cell that will weigh about a kilogram, and should, Müller says, provide enough energy to replace 13 kilograms of batteries.

Targeting industry, Angstrom has supplied 24-hour, hydrogen-powered flashlights for use by security guards. And MTI has retrofitted readers of radio frequency identification (RFID) tags with fuel cells, so that they can last a full eight-hour shift in a warehouse.

As fuel cells shrink and prices come down, the technology should make its way into mass consumer products such as phones and laptops. But some observers remain sceptical. Bradford of Frost and Sullivan wonders how willing consumers will be to buy refills for their fuel cells, when they perceive recharging from a wall socket as essentially free.

And Bryant predicts that fuel cells will go into niche applications — for industry and the military, and people who have to operate far from power outlets. He thinks that better energy efficiency in electronics together with constant, marginal improvements in existing power packs mean that — for most of us — batteries will remain good enough. ■

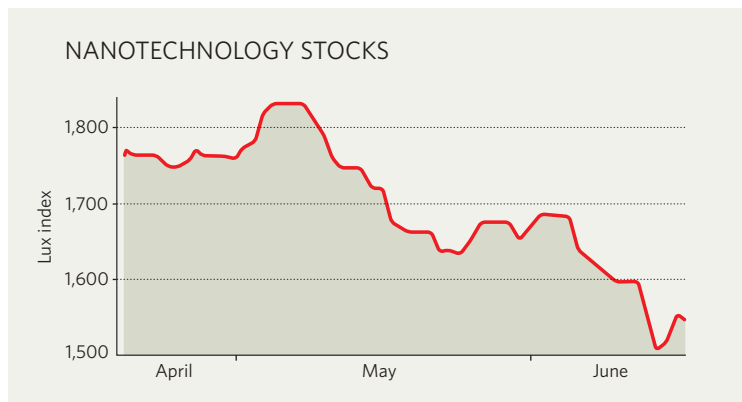
IN BRIEF

OUT OF CHIPS Philips, the giant Dutch electronics company, has said it will dispose of its semiconductor business by the end of this year. The company would like to float the business, which could be worth at least US\$6 billion, on the stock exchange, but may also sell it to a rival. Philips, which plans to concentrate on lighting and consumer electronics, is thought to have tired of the sharply cyclical nature of the chip sector. According to the California-based Semiconductor Industry Association, total chip sales slumped to \$139 billion in 2001, for example, but were back up to \$227 billion last year.

VIAGRA APPEAL A group of Chinese drug firms has appealed against a court ruling that blocks the companies from making generic copies of the impotence drug Viagra. The alliance of 13 firms is challenging a 2 June decision by a Beijing court that upheld Pfizer's patent on its blockbuster drug. That ruling overturned a 2004 decision by China's patent-review board that had sided with the generic makers. The case is being widely watched as an indication of the degree to which China will respect Western companies' intellectual-property claims (see *Nature* 440, 990–991; 2006).

ANTHRAX ACQUISITION A prominent biotechnology company that has ridden the wave of the genomic revolution for 14 years without selling a single product has finally found a customer. Human Genome Sciences of Rockville, Maryland, says that the US health department will buy 20,000 doses of its experimental anthrax drug, ABthrax. The \$165-million order is part of the department's \$5.6-billion Project BioShield. But the drug — a human antibody that blocks toxins released by anthrax bacteria — will first need regulatory approval.

MARKET WATCH



SOURCE: LUX RESEARCH

Stocks in companies associated with nanotechnology took a pounding in May as global investors ran away from risk. The market dived as part of the month's general stock-market dip, when fears of higher interest rates sucked money out of risky investments.

It is the overall market environment that has brought about the fall, says Peter Hebert, head of Lux Research in New York, which compiles a nanotechnology index (symbol LUXNI on the American Stock Exchange) from stocks in firms that sell nanotechnology, or rely on it. "Investors are concerned about interest rates," he says.

The steep drop in the index is, in fact, not much different from that in the Nasdaq, the benchmark for all high-technology stocks in the United States. And if you look back over Lux since it

started in 2003, the latest dip looks fairly typical of annual fluctuations that occur as enthusiasm for the sector waxes and wanes.

Some stocks still managed to do well. Massachusetts-based Nucrust Pharmaceuticals, for example, which makes spray-on bandages out of silver nanocrystals, has performed strongly. And stock in the Pennsylvania cell-analysis company Immunicon has shot up since March, on good financial results.

Even as major indices have stabilized after May's falls, Hebert notices that investor sentiment towards young nanotech companies remains "skittish". But he thinks those who hold tight will ultimately be rewarded. "There are strong fundamentals behind most of these businesses," he says. ■

Colin Macilwain

Seeing is believing as brain reveals its adaptability

SIR — It is traditionally accepted that acquisition of vision must occur in the first year or two of life, before the critical period for vision has elapsed. We were fascinated, therefore, by your News Feature “Look and learn” (*Nature* **441**, 271–272; 2006) reporting acquisition of competent vision in Pawan Sinha’s patient following almost 30 years of functional blindness, particularly as we have observed a similar phenomenon in the more limited domain of stereo-blindness.

Although stereo-blindness is infinitely less disabling than total blindness, it too is generally considered to be incorrigible if not treated in early childhood. Yet we have recently followed a 50-year-old stereo-blind woman who, with proper ocular alignment, has been able to achieve full stereopsis, including the perception of random-dot stereograms, after many decades of stereo-blindness (O. Sacks *New Yorker* 64–73; 19 June 2006).

A critical period for the development of many aspects of visual perception remains a valid concept, and every effort should be made towards early intervention when compromised vision is detected. Nonetheless, if there is any early vision at all — even such limited vision as Sinha’s patient had, or the very small fusional area that severe esotropic strabismus may permit, as in our own patient — it seems that islands of cortical function may be established, which can be reactivated and enlarged even decades later, given the requisite optical or surgical help. In such a situation, there is apparently enough cortical plasticity still present in the adult brain to allow, in some people, a full visual recovery. **Oliver Sacks*, Ralph M. Siegel†**

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Discrete reminder about Weismann’s discovery

SIR — Like any other science, genetics cherishes its stories about its heroes (“Hard to track” *Nature* **441**, 400; 2006). In his published papers (see www.mendelweb.org), and in his letters to Carl von Nägeli (C. Stern and E. R. Sherwood *The Origin of Genetics*; Freeman, 1966), Gregor Mendel makes no mention of discreteness; and he probably never thought about it.

Mendel was lucky that the discrete symbols of algebra agreed with the discrete units of inheritance. Mendel is the founder of genetics for discovering the algebraic laws of

biological inheritance, not discreteness. It was Ronald A. Fisher (*The Genetical Theory of Natural Selection*; Clarendon, 1930) who put Mendel and discreteness on the same page, creating the modern myth by implication.

The discreteness of the modern gene was described by August Weismann as early as 1893, in his classic book *The Germ Plasm* (Scribner’s), which is still in print. In it, Weismann describes units of inheritance, or ‘ids’, as ‘granular’. Fisher was aware of Weismann’s ids (R. A. Fisher *Science Progress* **21**, 159–160; July 1926), and probably wanted to shift credit to his fellow mathematician, Mendel.

In *The Germ Plasm*, Weismann also republishes a drawing by Theodor Boveri, a co-discoverer of meiosis, showing paired units of inheritance arranged in a double row, like beads on a string.

William L. Abler

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Researchers frustrated by lack of input to NIH policy

SIR — In the current discussion about the US National Institutes of Health (NIH), its director and its direction^{1–3}, we have heard much about the fact that funding will probably not increase for the foreseeable future, and about the many challenges confronting the organization.

Clearly, where there is adversity, there is opportunity. Thus, one cannot help but agree with Michael Bishop and Harold Varmus that the way forward lies in “making common cause with the leadership of NIH”² and joining forces. Unfortunately, an issue that has been overlooked is exactly how to make this happen.

Many NIH-funded investigators ‘in the trenches’ are very unhappy right now, not only because funding is tight, but also because they are frustrated by a lack of ready access to, or input in to, global funding decisions and important policies. I suspect that if there were more discussion about these critical issues, then investigators would be much more understanding and even at ease. Instead, it seems as though there is a wide communication gap between those who make decisions about funding (Congress and the NIH leadership) and those who do and direct so much of the research that the funding supports (typical scientists).

Many investigators are so busy with research, patient care and administrative duties that time for such exchange is limited. Nonetheless, both the NIH and the biomedical-research community would benefit from thoughtful, transparent

discussion about biomedical research in the United States in a structured forum. It could even lead to productive action.

Don C. Rockey

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1. Marks, A. R. *J. Clin. Invest.* **116**, 844 (2006).
2. *Science* **312**, 499 (2006).
3. *Nature* **441**, 17–19 (2006).

Public will fear biological accidents, not just attacks

SIR — As one of the few Europeans who took part in the Synthetic Biology 2.0 conference in California in May, I read your Editorial “Policing ourselves” (*Nature* **441**, 383; 2006) and News story “Synthetic biologists try to calm fears” (*Nature* **441**, 388–389; 2006) with great interest. The impression I received is that synthetic biologists are trying to alleviate public concerns over their research area — but will have serious difficulty in succeeding.

Most discussions in the field about self-regulation are focused on biosecurity — that is, preventing new opportunities for bioterrorists — in an attempt to act proactively and prevent overly restrictive regulations being imposed by the authorities. But the concerns raised in the open letter from civil organizations including Greenpeace and Genewatch UK deal with the biosafety aspects: in other words, the uncertainties and unintentional consequences of synthetic-biology research, as opposed to its deliberate misuse. Although biosafety concerns were discussed at the conference, they are mainly unaddressed in the self-regulation attempt. Yet they will be increasingly significant as the synthetic-biology field develops, especially in Europe.

Synthetic biology shares many characteristics with other new technologies regarding public perception of novelty, uncertainty and controllability; we are all aware of the controversy in Europe over genetically modified crops. This time, we should be more far-sighted, and proactively address biosafety concerns as well as ethics, and intellectual-property rights as much as biosecurity. The Synthetic Biology 3.0 conference in Zurich next year will be a good opportunity.

Markus Schmidt

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Correction

The name of the co-author, John Lancaster, was accidentally left off the Correspondence letter “‘Referee factor’ would reward a vital contribution” (*Nature* **441**, 812; 2006).

BOOKS & ARTS

Faster, better, healthier

An exploration of how genetics has influenced many aspects of life.

The Strongest Boy In The World: How Genetic Information Is Reshaping Our Lives

by Philip R. Reilly

Cold Spring Harbor Laboratory Press: 2006. 278 pp. \$29

Michael A. Goldman

Physician, scientist, lawyer and chairman of healthcare company Interleukin Genetics, Philip Reilly has written five books on human genetics. Although his last book, *Is It In Your Genes?* (Cold Spring Harbor Laboratory Press, 2004), was targeted at health professionals, *The Strongest Boy in the World* is more in the vein of his earlier ones, being aimed at a broad, general audience.

The Strongest Boy in the World is a wonderful tour of genetics, genomics and stem-cell biology. General readers may find the science a stretch, but the effort will be amply rewarded with a better understanding of some of the most important issues currently facing our society. It isn't that Reilly gives new perspectives, but rather that he presents a rich, fascinating history and a broad view of the science that seasoned geneticists think about every day. Instruction about basic principles of genetics is minimal, with a 'knock-out' mouse being defined in terms of a transgenic mouse, for readers who know what the latter is. Reilly delves into broad fields of biology, society and history, clarifying the idea of 'race', but rather muddying the term 'family'.

The first part of the book explores what it means, in the light of our knowledge of genomics, to be human. The title essay, "The strongest boy in the world", refers to a child in Germany born into a family known for exceptional strength. The boy had a mutation in the myostatin or growth differentiation factor 8 (GDF-8) gene, which suppresses muscle growth. The child was an extreme variant, with quadriceps more than six standard deviations above the mean and an exceptionally low body-fat content. Drugs that inhibit the myostatin gene are currently being studied to alleviate the muscle-wasting symptoms of diseases such as cancer and AIDS, but it is only a question of time before such drugs are used for enhancement as well as treatment.

Reilly moves on to a broad-ranging discussion of athletic ability and the extent to which it might be influenced by heredity as well as the environment. He cites specific examples of



T.A. CLARY/AFP/GETTY

Running in the family: did genetics help Robert Cheruiyot and other Kenyans win the Boston marathon?

genetic variants that can or should have an effect on athletic performance. On this topic, Reilly is not judgemental: "I am not sure where I stand on the whole issue of efforts to 'enhance' performance. Elite athletes devote most of their lives to trying to do just that." Is it so different from steroid doping, or hiring an expensive trainer or orthopaedic surgeon? Or, for that matter, exercising? Should a child born with a natural mutation that greatly enhances athletic ability be allowed to compete? "There is no question that genes greatly influence athletic ability ... gender is determined by genes, and there is a significant gender gap in athletics."

There is also, Reilly points out, a gap in athletic ability between some ethnic groups. Kenyan athletes, who largely come from ethnic groups representing only 0.05% of the world's population, have won 14 of the past 16 Boston marathons, and it isn't just due to diet, tradition and altitude. Reilly describes, in limited detail, specific enzyme variants that seem to confer a benefit in endurance events, and others

that may be advantageous in sprinting. But he is cautious: "It would be foolish and wrong to suggest that one group of people is in some way genetically superior to other groups." He does suggest, however, that some people, in addition to other qualities, "may have been born with gene variants that by chance support their goals". Reilly sees a time when gene testing may be part of a physical examination for professional athletes, predicting who is most likely to succeed and who might be at risk of premature death. But he doesn't address questions such as how we draw the line between genetic traits or tendencies that can be used in screening for fitness for employment and those that cannot.

In four more engaging essays, Reilly goes on to explore human origins, race, longevity and intelligence, in each case looking carefully at the influence of genetics, and what our understanding of genetics means for our view of ourselves. Throughout the book, each chapter stands alone; this results in some repetition of

ideas, but it has the tremendous advantage that the reader can dive into any section of interest and feel at ease.

The second part of the book, on diseases, is a random walk through five different genetic conditions. This allows Reilly to explore different aspects of medicine, different disease aetiologies, different modes of therapy, and the complex ethics and sociology of conditions such as deafness, in which non-hearing parents may actually want to have non-hearing children. Missing from Reilly's treatment is a deeper look at genetic mechanisms. Trinucleotide repeats are introduced as more or less incidental to Huntington's disease, rather than as part of a set of more than a dozen disorders with an astoundingly similar underlying mutation mechanism that involves rapid changes in highly unstable stretches of DNA.

The third part of the book is an investigation of dogs, cats, mice, corn and rice, what genetics has meant for them, and what these organisms have meant for the development of modern genetics. Reilly's treatment of corn is typical of his approach to other organisms. He writes about corn's place in the human economy, its domestication and breeding, and what we have learned about its genetics. He then analyses some of the work on genetically modified corn, addressing a topical issue that could have profoundly disturbing implications for ecology and food safety — or that could lead to fantastic gains in the war on hunger. Reilly does not ignore the arguments against the use of genetically modified foods. He even sympathizes with those who fear multinational agriculture companies taking over the world at the expense of small farmers. But there's no question how Reilly feels about this technology. "I side squarely with those who favor the rapid, safe development of genetically modified groups. I think it could be the most important positive event for the environment in history."

The final section deals more specifically with the implications of genetic and reproductive technologies for our society. Reilly addresses not only the obvious topics such as stem-cell biology, preimplantation genetic diagnosis, and forensics, but also what we can learn about history through genetics (tracing US president Thomas Jefferson's genes, for example), and how genetics has come to pervade our art and language.

For the geneticist, Reilly presents a balanced, positive view of ethical and social issues in genetics, and an entertaining background in history, geography and economics, and the way these fields interface with modern genetics and genomics. I've often tried to convince my colleagues across campus that genetics should be a part of every undergraduate's education. No book makes this case more clearly than *The Strongest Boy in the World*. ■

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A glimpse of the early Universe

Chasing Hubble's Shadows: The Search for Galaxies at the Edge of Time

by Jeff Kanipe

Hill & Wang: 2006. 224 pp. \$24

Patrick Petitjean

Can beauty save the world? Anyone who reads *Chasing Hubble's Shadows* by Jeff Kanipe may be able to find an answer in the book's images of a tiny part of the sky, taken by the Hubble Space Telescope. They reveal thousands of galaxies, the "Hubble's shadows" of the title, at different stages of their evolution. The images portray breathtaking beauty surrounded by silence.

The book is about the beauty of the heavens as revealed by Hubble, the questions such images raise, and the explanations astronomers have proposed, along with their doubts and hopes. How did astronomers become interested in galaxies? How can galaxies be classified? How are the most remote of them detected? How can their formation and evolution be explained? The book is much more than a description of the farthest images ever taken. It is also a vivid description of how astronomers work and how ideas develop. It is a virtual trip into the complex realm of dark matter and dark energy, but also a real trip through the Moon-like landscape of the Mauna Kea summit in Hawaii where the world's biggest telescopes are located. This is an exciting time, with astronomers facing challenges similar to those that confronted Galileo when he discovered the satellites of Jupiter. Astronomers are trying to understand the physical processes at play in the early Universe.

Kanipe has written a narrative rather than an analysis. He reports discussions with dozens

of astronomers and their explanations of a variety of subjects relevant to the early Universe and to the formation and evolution of galaxies. The writing is uneven and a few topics are discussed several times, but isn't the art of saying things several times without bothering the reader part of pedagogy? The resulting mixture of journalistic and scientific styles certainly keeps the reader excited.

The book will not help readers understand physics from first principles, or discover a theory that can make sense of the little information we have on our Universe. Rather, it provides an opportunity to meet people and ideas. This is an entertaining way to approach the relation between the big scientific questions and the emotions that overcome astronomers during their research. We hear of their nightmares, their doubts, their patience, their scepticism and the risks they take.

It is difficult to define the target audience for this book. There are sometimes too many inappropriate technical details for a general reader and, probably driven by his enthusiasm, the author also goes too fast or too far. For example, it may be unrealistic to assume that general readers know what a graviton is, or why it may be interesting to follow up on radio galaxies, and specialists will learn little too. Nonetheless, if general readers are happy to leave a few technical details unexplained, they will be able to enjoy this entertaining approach to the mysteries of our Universe. Anyone curious about the mechanisms of our world will be thrilled by the book.

Few would disagree with the remarks here concerning the lack of funding, although these are hardly unique to astronomy. Kanipe also questions the political choices being made,



The Hubble Space Telescope has identified infant stars in the Small Magellanic Cloud galaxy.

NASA/ESA/A. NOTA

such as the US plan to visit the Moon and Mars. It would have been more appropriate to question the place of science and basic research in our society. This has to be carefully argued to convince a broad audience, but I am afraid he is preaching to the converted.

The main aim of the book is to describe what astronomers have learned so far about the first stars and galaxies in the early Universe, but it does much more than this. Given the huge strides being made in exploring the Universe, physicists are having to find new and different

ways of explaining what they see. This creates the feeling that we have reached the end of the beginning and are looking ahead. ■

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B. GILDEN/MAGNUM PHOTOS



Glass distinction: it's all in the flavour, the colour and the bouquet for those who wish to enjoy wines — or identify them in a 'blind' tasting.

A question of taste

Molecular Gastronomy: Exploring the Science of Flavor

by Hervé This (transl. by M. B. DeBevoise)
Columbia University Press: 2006. 360 pp.
\$29.95

John Piggott

In the introduction to this book, Hervé This says what he means by 'molecular gastronomy', explains its origins and carefully differentiates it from food science. In essence, he defines molecular gastronomy as the science (physics, chemistry and biology) of enjoying food. However, insofar as it is science applied to food, it is simplest to regard it as a branch of food science, concerned particularly, although not exclusively, with the transformation of ingredients to foods and their subsequent consumption. He applies it especially to culinary, rather than industrial, processes.

Molecular Gastronomy is an anthology of columns from *Pour la Science* (the French edition of *Scientific American*), mostly published in the 1990s. The short chapters are organized into four sections. The first part describes investigations and explanations of traditional cooking methods, and the second covers the physiology of flavour (mostly taste). The third and longest part is composed largely of discussions of journal articles and news stories, with most being explanations of how traditional products work. Finally, applications of recent science and technology are discussed

to suggest new products derived from existing ones. At the end there is a glossary, a list of further reading and a good index.

One or two of the explanations in the first section are not convincing but would make a good starting point for anyone wanting to take them further. Elsewhere the descriptions and explanations of recent science are clear and coherent but uncritical. For example, he cites a statistical analysis of some Scotch whisky tasting data that demonstrates only that if you use inappropriate data in a statistical analysis you get meaningless results. There seems to be disproportionate attention paid to alcoholic beverages, including a chapter about warming and chilling wine. This did not go far enough for me; there is no help with practical problems, such as how long you should leave a bottle of white wine or champagne in the freezer to cool it quickly, and whether it matters. Or how long a bottle of red wine left overnight at -5°C and needed for lunch should be left in the microwave oven and at what power?

Most of the work discussed was done in France, but maybe only laboratories there work on products of interest to a French audience, or perhaps they are better at publicity. It is both a weakness and a strength of this book that it is French. I cannot imagine anyone being funded to do this work in Britain or the United States, where there is less interest in food, but at least someone is doing it. Surely no one outside France cares whether the yolk

is exactly in the middle of a boiled egg, and the English-speaking world, which is presumably the target for this edition, probably cares even less than the rest. In fact it is hard to see where the market for this book would be. Reading these intelligent but light-hearted columns in a monthly magazine is one thing, but buying and reading the book is quite another. Many of these stories date from 10 to 15 years ago, which is regarded as history by several of my colleagues, and even in food science the work is likely to have been overtaken by events.

The translation is generally very good, and the book makes a worthy attempt to explain the science to non-specialist readers. There are a few mistakes, but not important ones. For example, it refers to Brussels sprouts as a root vegetable — have I been eating the wrong bit all these years? In any case, the original sources are listed as 'further reading', although the individual chapters do not cite the references.

There is too much science to make this a mass-market book, and it is certainly not for the specialist food scientist. It would make a good present, perhaps, for a 'foodie' scientist, and I would put it on the reading list for an introductory food-science course, as it might interest some of the students. ■

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Correction

The Science in Culture "Home from home" (*Nature* **441**, 816; 2006) by Colin Martin should have said that Benjamin Franklin died in Philadelphia, rather than at Passy near Paris.

QUANTUM PHYSICS

Atomic gas in flatland

Tilman Esslinger and Gianni Blatter

The observation of Bose–Einstein condensation in an atomic gas was a seminal result. Two-dimensional gases are more complex, and an intriguing interference experiment has exposed a different superfluid transition.

Atomic quantum gases are unique systems for investigating elementary concepts in many-particle physics. The most prominent example is Bose–Einstein condensation — the formation of a gaseous superfluid through the collapse of an ensemble of atoms into a single quantum state — which was first observed in an ultracold gas of rubidium atoms¹. Atomic gases are useful because their ingredients are familiar: the atoms interact through well-defined two-body collisions, and the trapping potentials confining the gas are known. That simplicity allows experimental insights to be linked directly to theoretical fundamentals.

But can physics in two dimensions — which is intrinsically different from that in three — also be investigated in atomic quantum gases? Hadzibabic and colleagues set out to do just this, and their results are published on page 1118 of this issue². In an intriguing experimental set-up, the authors used laser-induced optical potentials to squeeze an ultracold ‘Bose’ gas — consisting of atoms with integer spin, in this case rubidium-87 — into flat traps so that the atoms could move only within a plane. To find out what the gas was doing, they cunningly placed two traps on top of one another and released the atoms from both. Images of the resulting interference patterns yielded information on crucial aspects of the two-dimensional gas³.

The history of physics in two dimensions is littered with puzzles and surprises. In 1966, a theorem proclaiming the absence of a class of phase transitions in one and two dimensions was proved^{4,5}. Essentially, minute thermal fluctuations prevent order from emerging over large distances in such systems. A Bose–Einstein condensate consists of atoms whose quantum-mechanical wavefunctions are ‘coherent’ — perfectly in phase — and so exhibit a form of long-range order. Thus, an immediate consequence of this theorem was that, except purely theoretically at a temperature of absolute zero, a Bose–Einstein condensate cannot exist in one or two dimensions.

In two dimensions, however, the destruction of order by thermal fluctuations is only marginal: order can develop over short distances,

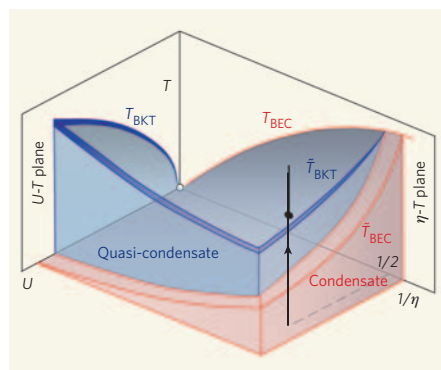


Figure 1 | Qualitative phase diagram for a two-dimensional trapped atomic Bose gas. T is temperature, U collisional interaction strength. The trapping parameter, η , characterizes confinement: an infinite value of η (so at 0 on the $1/\eta$ axis) corresponds to the flat system with steep walls, and $\eta = 2$ (marked at the point $1/\eta = 1/2$) describes a harmonically curved trap. On the U – T plane at $1/\eta = 0$, a homogeneous gas will undergo a Berezinskii–Kosterlitz–Thouless (BKT) transition at T_{BKT} into a superfluid phase, but a Bose–Einstein condensate (BEC) cannot form. The thickness of the blue line indicates the size of the jump in superfluid density at the transition point. The η – T plane at $U = 0$ describes a trapped ideal (non-interacting) gas with a Bose–Einstein crossover, at T_{BEC} , into a condensed phase that is not superfluid, with no BKT transition. Away from these planes, two crossovers are expected: at $\tilde{T}_{\text{BKT}}$, a sharp crossover takes the system into a quasi-long-range ordered phase with finite superfluid stiffness (blue region). The broad crossover at the lower temperature $\tilde{T}_{\text{BEC}}$ marks the onset of order throughout the entire trap, establishing a condensate with a finite superfluid response. Hadzibabic and colleagues’ experiments² were carried out on the black line. On increasing the temperature, they observed both the disappearance of quasi-long-range order and the appearance of vortices (black dot).

decaying slowly with increasing distance so that quasi-long-range order survives. This reduced order is sufficient to generate another property of condensed matter: that of stiffness, such as occurs when a solid resists the shear force applied to two opposite surfaces of the body. This shear stiffness is lost when the solid

melts and becomes a liquid. Analogously to the behaviour of a solid, below a certain temperature a Bose gas resists a ‘twist’ in the phase of its atoms applied to its boundaries. This phenomenon is the prerequisite for establishing a superfluid that flows without friction.

Usually, such stiffness is established by a phase transition into an ordered phase. But in two dimensions, stiffness can be present without true order. The Soviet physicist Vadim L’vovich Berezinskii was the first to understand the curious nature of this superfluid yet non-ordered, low-temperature phase. In 1971, he predicted the existence of a phase transition⁶ that he attributed to the uncoupling of bound pairs of vortices with superfluid currents circulating in opposite directions⁷. Some months later, Michael Kosterlitz and David Thouless^{8,9} presented an elegant thermodynamic argument that established the correct expression for the temperature, T_{BKT} , at which this Berezinskii–Kosterlitz–Thouless (BKT) transition occurs. Going above this temperature, quasi-long-range order suddenly disappears, as does superfluidity — the superfluid density collapses to zero in a characteristic jump that depends only on T_{BKT} and the mass of the atoms involved¹⁰.

The particular case relevant to the work of Hadzibabic *et al.*² — a two-dimensional Bose gas whose atoms interact only weakly — has been studied in detail¹¹. Recent quantitative numerical work¹² has established the precise location of T_{BKT} for the superfluid transition in such a gas as a function of the strength of interaction, U , between its atoms (the U – T plane in Fig. 1). As U diminishes, the superfluid density decreases and T_{BKT} approaches zero. This projected trend agrees with the observation that a non-interacting gas ($U = 0$) does not support superfluidity.

The question of whether the finite room for movement that is induced by a trapping potential such as that of Hadzibabic *et al.*² changes the physics of a non-interacting Bose gas has also been analysed¹³. The dramatic conclusion was that such a confinement modifies the density of allowed states such that a crossover to a Bose–Einstein condensed (and

hence ordered) phase is also possible in one- and two-dimensional traps. By considering trapping potentials with the algebraic form R^η (where R is the radial distance from the centre of the trap and η is a parameter indicating the steepness of the trap's walls), the authors established a continuous connection between a 'harmonic' trap (with $\eta = 2$) and a trap with a flat bottom and extremely steep walls ($\eta \rightarrow \infty$). This latter limit mimics the behaviour of a homogeneous gas of finite density and, as expected, the transition temperature to a Bose–Einstein condensate, T_{BEC} , goes to zero as η increases (the η – T plane in Fig. 1).

A full description of Hadzibabic and colleagues' experimental situation further requires the inclusion of a non-zero collisional interaction ($U > 0$), as well as the harmonic trapping potential. In such a set-up, below a crossover temperature, $\tilde{T}_{\text{BEC}}$, the decrease of phase order with distance can be so slow that phase coherence is preserved over all atoms of the sample, and a Bose–Einstein condensate forms¹⁴. Above $\tilde{T}_{\text{BEC}}$, larger phase fluctuations destroy the condensate, but should still leave the system in a superfluid state with quasi-long-range order. Hence, a second crossover at which superfluidity disappears when vortex pairs unbind must be expected at a higher temperature, $\tilde{T}_{\text{BKT}}$. The consistency of this picture (Fig. 1) is supported by a vanishing jump found in the superfluid stiffness when approaching the ideal (non-interacting) gas limit¹⁵, and by numerical simulations¹⁶.

Hadzibabic and colleagues² carried out their experiments along the black line in the phase diagram of Figure 1. They analysed the contrast in the interference patterns of atoms emerging from their two traps as a function of the spatial range². With increasing temperature, they identified a sudden decrease in the degree of phase order, indicative of a jump in the superfluid density at $\tilde{T}_{\text{BKT}}$. Simultaneously, the interference patterns were flooded with dislocation lines (Fig. 4 on page 1120), signalling the presence of vortices¹⁶.

The jump in density and flooding with vortices appeared over an appreciable range in temperature, as would be expected for a transition phenomenon in an inhomogeneous sample. Caveats do remain, however. The temperature range over which the jump was observed has yet to be related to the trap configuration, and the second smooth crossover into the Bose–Einstein condensed phase, which should be fully coherent over the entire sample size, has not been observed. Nevertheless, Hadzibabic and colleagues' direct observation² of the Berezinskii–Kosterlitz–Thouless transition shows once again how fundamental concepts of physics can be extracted from experiments in a quantum gas. ■

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STRUCTURAL BIOLOGY

RNA switches function

Steve Reichow and Gabriele Varani

Proteins are not the only regulators of metabolite synthesis — some RNA molecules do it too. These RNAs lack chemical diversity, so how do we explain the variety of their respective substrates?

The traditional view of RNA as a passive messenger in the transfer of genetic information has long been abandoned. Yet every few years we are still surprised by the discovery of another function that RNA performs. Some bacterial messenger RNAs directly regulate the expression of proteins involved in the synthesis of certain metabolic products. This regulation responds to changing levels of the metabolites in the cell, a type of feedback mechanism that was previously known to be mediated only by proteins. These RNAs — known as riboswitches — elegantly couple metabolite recognition with gene regulation in the absence of protein helpers. In this issue, Serganov *et al.* (page 1167)¹ and Montange and Batey (page 1172)² describe the structures of two distinct classes of riboswitch, providing remarkable insight into RNA metabolite recognition and the control mechanism for gene expression.

Riboswitches are not rare — they account for about 2–3% of genetic control in bacteria³, and are also found in archaea, fungi and plants⁴. These regulatory RNA domains are typically located in regions of mRNA that directly precede the protein-coding sequence. They use a simple modular architecture — a metabolite-sensing domain recognizes the substrate, and another region, known as the gene-expression signal, regulates protein production in response to substrate binding. When the substrate binds to the metabolite-sensing domain, a structural reorganization of the RNA occurs that unveils (or sometimes masks) the gene-expression signal. In many cases, the end result is suppression of the production of enzymes that are responsible for the biosynthesis of the detected metabolite (Fig. 1).

When riboswitches were first discovered, two questions immediately arose: how is the metabolite recognized, and how does binding

of the substrate trigger genetic regulation? Preliminary answers were provided by the structures of guanine- and adenine-sensing riboswitches^{5,6} — guanine and adenine are organic bases collectively known as purines. The most astonishing finding was the extraordinary selectivity achieved by these RNAs: for instance, the guanine-sensing riboswitch binds to guanine with 100,000-fold greater affinity than it does to adenine, a remarkable feat considering the close chemical similarity of the two purine substrates. Amazingly, the strict selectivity is completely reversed by the substitution of only a single RNA base in the binding site. This exquisite molecular recognition is accomplished by completely encapsulating the substrate in the riboswitch, surrounding the substrate with specific RNA contacts.

The two structures presented in this issue^{1,2} provide even more remarkable examples of RNA molecular recognition. Serganov *et al.*¹ describe a riboswitch that senses thiamine pyrophosphate (TPP, an enzyme cofactor derived from vitamin B₁) in bacteria. The structure of a TPP-sensing riboswitch in plants has also been reported elsewhere⁷. Montange and Batey² study a riboswitch that binds S-adenosylmethionine (SAM, a cofactor that donates a single carbon unit and that is required for many enzymes). Both TPP and SAM are chemically and structurally more complex than purines. Notably, TPP carries two negatively charged phosphate groups. In contrast, molecules that bind to RNA are typically decorated with positively charged groups that provide favourable electrostatic interactions with the negatively charged RNA.

The TPP-sensing structures^{1,7} show that RNA recruits positively charged metal ions to mediate otherwise unfavourable electrostatic interactions, resembling the use of metal ions

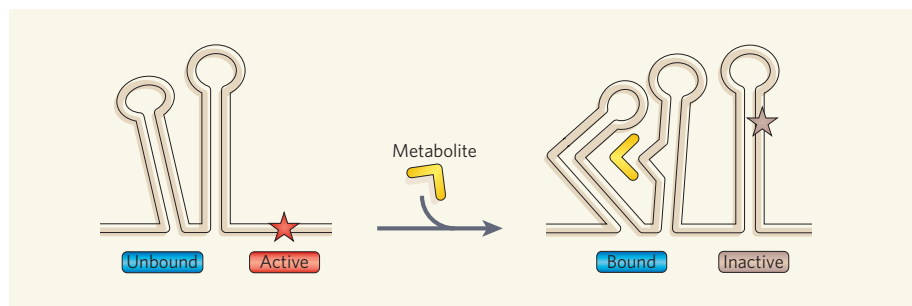


Figure 1 | Gene regulation by riboswitches. The RNA of a riboswitch contains two functional domains: a metabolite-sensing domain (blue) and a gene-expression signal (green). These domains adopt interdependent conformations in response to the presence or absence of a particular metabolite (yellow). In this example, the gene-expression signal is required for the initiation of protein synthesis. When the metabolite is absent, the metabolite-sensing domain adopts a conformation that reveals the gene-expression signal and allows protein synthesis to occur (indicated by the red star). When the metabolite binds, the ensuing structural reorganization leads to the sequestration of the gene-expression signal, shutting off protein production (indicated by the grey star).

to glue together other RNA structures⁸. Just like guanine or adenine in the purine-sensing riboswitches, TPP and SAM become deeply buried in the RNA structures, embedded between interacting helices. The similar mode of substrate recognition observed in diverse riboswitch classes suggests a common functional mechanism, whereby conformational adaptability enables the RNA to encapsulate the substrate.

Highly specific substrate recognition by proteins exploits an arsenal of 21 amino acids that vary significantly in charge, size and polarity. By contrast, RNA has only four components at its disposal — adenine, guanine, cytosine and uracil — that are all similar in size and chemistry. The riboswitches described in this issue^{1,2} demonstrate how RNA overcomes its limited chemical diversity by adaptively folding into remarkably complex architectures to create highly specific binding pockets. The overall architectures created by these two riboswitches are surprisingly similar and resemble small RNA enzymes (ribozymes).

The close structural similarity of riboswitches from bacteria¹ and plants⁷ emphasizes how difficult it may be for a single RNA molecule to combine substrate recognition with genetic control. This similarity has fuelled interest in riboswitches as attractive targets for antibacterial drugs, particularly because there is an apparent absence of such RNA systems in humans. Indeed, a TPP analogue (pyrithiamine pyrophosphate, PTPP) shows antimicrobial activity by blocking the binding site of the TPP-sensing riboswitch, shutting down thiamine metabolism in bacteria.

Unfortunately, these RNAs may be surprisingly proficient at developing drug resistance. Microbes develop resistance to PTPP by allowing mutations to emerge within the riboswitch, despite the need to preserve the RNA's structural architecture. A single mutation is sufficient to override the inhibitory effects of the drug, rescuing thiamine synthesis^{1,7}. It seems that the structural flexibility that allows a

riboswitch to alter its conformation upon substrate binding may also facilitate the emergence of resistance to drugs targeted against it.

Since their discovery a few years ago, numerous riboswitches have been found that specifically recognize substrates as diverse as nucleotides, amino acids, vitamins and co-enzymes⁹. These RNAs are evolutionarily ancient, and are widespread in bacteria, plants

and fungi. Studies of aptamers — RNAs selected experimentally to recognize small molecules — demonstrate that RNA can discriminate between closely related structures at least as well as antibodies can¹⁰. It is now clear that natural selection can also produce RNAs with extraordinary substrate specificity. Does this mean that small-molecule drugs could be found that bind to these RNAs and inhibit their function? Would bacteria and fungi become resistant to such drugs, or do riboswitches represent a chink in microbial armour?

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MATERIALS SCIENCE

Germanium takes holey orders

Andreas Stein

Soap-like molecules serve as a scaffold for remarkably well-ordered, porous germanium skeletons. The nanometre-sized features of these semiconductor frameworks confer unique optical and electronic properties.

As semiconductor materials are shrunk to the nanoscale, their physical properties begin to alter: colours change, melting points decrease, electron energy bands turn into discrete levels, and reactive surface areas become proportionately larger as particle size decreases¹. This applies not only to discrete semiconductor particles, but also for extended 'mesoporous' framework structures of nanometre scale. In this issue, two papers^{2,3} detail approaches to the synthesis of periodic porous structures made of germanium. The optical properties of these materials are shown to depend on their dimensions, composition and the presence of other molecules attached to their surface.

Porosity can be an extremely useful characteristic in a material, with the attendant large surface area benefiting applications in which molecules interact with a surface, such as sensors and catalysts. Silicon with nanoscale holes has been widely studied since its luminescent properties were discovered⁴. But porous forms of a fellow member of group 14 of the periodic table — named ekasilicon by Mendeleev, but

rechristened germanium by its discoverer, Clemens Winkler, in honour of his home country — have been investigated much less. Germanium's interest lies in its semiconducting nature and use in transistors, and also in its applications as a component of fibre-optic cables, as the focusing element in infrared-sensitive night-vision systems and as a polymerization catalyst in the manufacture of soft-drinks bottles. Size-dependent properties can also be achieved with germanium at relatively large feature sizes, and, in a mesoporous form, unusual electronic and optical properties are to be expected.

But few procedures exist to synthesize porous germanium. Etching and vapour-deposition techniques have been used to form germanium films with somewhat random pore structures and feature sizes^{5,6}. Now, Armatas and Kanatzidis (page 1122)² and Tolbert and colleagues (Sun et al., page 1126)³ describe the use of a versatile technique known as surfactant templating to synthesize mesoporous germanium structures with

cubic and hexagonal geometry, respectively.

Surfactant templating involves the self-assembly of inorganic building-blocks through their electrostatic interactions with organic 'amphiphilic' surfactant molecules that, like those in soap, have both hydrophilic and hydrophobic properties⁷. According to how these interactions vary, different geometries are obtained as the inorganic components link to form a framework. Forms of silica with well-ordered cubic or hexagonal channel systems, or with disordered worm-like tunnels or layered structures, have already been synthesized using this technique^{7,8}. In these mesostructured materials, inorganic regions alternate with organic template regions at typical distances of several nanometres. To obtain a mesoporous solid with accessible channels and large surface areas, the organic template must be removed, often by combustion, without the inorganic framework collapsing. If this procedure works, it results in a structure that, although not necessarily crystalline on the atomic scale (mesoporous silica, for example, has amorphous walls), often shows regular order on the scale of the templated pore size.

The successful synthesis of mesostructured germanium must address several challenges. Chief among these is finding a soluble germanium precursor that self-assembles in the presence of an amphiphilic surfactant and that can be linked into a continuous framework. Self-assembly is itself promoted by the choice of a solvent that forms a hydrogen-bonded network but does not react with the precursor. The current papers^{2,3} use precursors that, although different, are both related to the compounds formed when highly electro-positive metals combine with elements from the middle of the periodic table⁹. These compounds were pioneered by Eduard Zintl in the 1920s and 1930s. That of Armatas and Kanatzidis² has the chemical formula Mg_2Ge and contains Ge^{4-} units in a crystalline lattice, whereas that of Tolbert and colleagues³, K_2Ge_9 , consists of more complex $(\text{Ge}_9^{2-})_n$ polymer chains derived from Ge_9^{4-} cluster ions.

To form an ordered, mesostructured germanium, Armatas and Kanatzidis add GeCl_4 to their reaction mixture to provide Ge^{4+} bridges that connect the Ge^{4-} units of the precursor. Tolbert and colleagues, on the other hand, link up their germanium chains by mild oxidation, taking care to avoid germanium oxide phases that would result from over-oxidation. The end result is, in the first case, cubic channel structures² and, in the second, two-dimensional, hexagonal channel structures³.

As in the case of mesoporous silica, the walls of both materials are amorphous on an atomic scale, but periodic on the mesoscale of the frameworks themselves. As anticipated, the materials exhibit size-dependent effects that result from so-called quantum confinement, a phenomenon that affects the allowed energy values of the material's electrons at smaller scales. In particular, compared with spectra in

bulk germanium, substantial shifts towards shorter, 'bluer' wavelengths are observed in the optical absorption or luminescence spectra of 1-nm-thick germanium walls.

A final challenge was the removal of the surfactant without destroying the mesostructure or converting germanium to its oxide. In the system derived from Mg_2Ge , this proved to be very difficult: Armatas and Kanatzidis state that "attempts to remove the surfactant from mesostructured germanium by thermal decomposition led to contraction of the inorganic framework with consequent loss of pore ordering"². Interesting properties are, however, observed in this system even without surfactant removal. Its optical bandgap — a crucial parameter in determining the conduction properties of a material — could, for instance, be smoothly varied through controlled partial oxidation and the generation of sub-oxide GeO_x species in the framework.

In the (Ge_9^{2-}) -based system, mild oxidation followed by an ion-exchange step generates cross-linkages in the $(\text{Ge}_9^{2-})_n$ that allow surfactant removal to take place. This results in a large surface area of 500 m² per gram of material, and the opened structure permits incorporation of guest molecules that shift the energies of electrons at the semiconductor's surface and so can modify its conductivity.

These papers^{2,3} both represent significant advances in the structuring of germanium frameworks. In a sense, the papers are complementary: they show the versatility of Zintl-compound chemistry in combination with surfactant templating and provide different avenues towards designed, ordered semicon-

ductor geometries. Tolbert and colleagues³ illustrate a way to produce true mesoporosity; Armatas and Kanatzidis² show that a cubic structure with continuous walls and continuous channels can be achieved, which is often a more desirable architecture to facilitate the access of guests to the whole pore system.

This work will no doubt spawn new mesoporous semiconductor systems of relevance in sensing, detection and communications. Opportunities exist for tuning the properties of these systems with mixed compositions, including the mixed Ge/Si systems³ and Zintl clusters involving other group-14 elements, or by using other linking molecules to connect Zintl compounds using Armatas and Kanatzidis's method. With the precedents of structural and compositional variety set by mesoporous oxides, the stage is set for mesoporous semiconductors with properties that can be engineered. ■

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ASTRONOMY

Supernovae brought to light

Carl Heiles

The existing catalogue of Galactic supernova remnants contains only a small fraction of the true number of these stellar explosions. A different observational technique is being employed to find the missing ones.

Just as nuclear bombs on Earth leave their legacy of devastation, stellar explosions — supernovae — leave their mark on the cosmos in the form of supernova remnants (SNRs) in the interstellar gas. The rate of supernova explosions in our Galaxy can be inferred from historical records of visual sightings and through comparison with other galaxies. These data, together with theories of SNR expansion and evolution, allow a prediction of the number of SNRs that should currently reside in our Galaxy. The result is tens of times larger than the number of known remnants, which are catalogued almost exclusively from the radiation they emit at radio frequencies¹. Writing in *The Astrophysical Journal*, Koo,

Kang and Salter² use a new technique to identify the more numerous older SNRs, which were missing from the catalogue because they are radio-dim.

The legacies left by supernovae, extending beyond cosmology, are often unappreciated. First, there is the anthropocentric point that we wouldn't be here without the elements, such as iron and carbon, that supernovae have caused to disperse into the interstellar gas. Over Earth's lifetime, several dozen nearby supernovae (within a distance of around 30 light years) would also have seriously modified Earth's environment, each for several hundred years³. The most serious consequences would have been the destruction of the ozone layer and a

great increase in the flux of highly energetic cosmic rays. That would have led to direct biological effects through increased radiation, as well as probable climate change that could dwarf today's global warming. It's even conceivable that greatly enhanced radioactivity emanating from a nearby supernova triggered the formation of life on Earth by producing complicated molecules⁴.

Supernovae have also greatly affected science and scientific culture. A supernova of 1572 landed the astronomer Tycho Brahe a job with King Frederick II of Denmark, an appointment that jump-started the principle of accurate astronomical measurements⁵. Tycho's protégé, Johannes Kepler, introduced and extended critical thinking and rigorous analysis as the basis for scientific endeavour⁶. Moving into the twentieth century, in seeking an explanation for the origin of a supernova, Walter Baade and Fritz Zwicky correctly predicted the existence of neutron stars⁷. More recently still, distant supernovae have been the signposts revealing the increasing acceleration of the Universe and the 'dark energy' that is held responsible for this phenomenon^{8,9}.

Supernovae come in two basic types, I and II. Type I supernovae are binary white dwarf systems, in which mass is transferred from an active companion star to a white dwarf (a low-to-medium mass star that has exhausted its nuclear fuel); type II supernovae are massive stars near the end of their lives. In both cases, a very dense object — the white dwarf or the core of the massive star — grows until it exceeds a critical mass, known as the Chandrasekhar limit. At this point, its internal pressure becomes unable to resist the force of its gravity, and it collapses in size by a factor of perhaps 1,000. That process releases a total of about 10^{46} joules of energy, the lion's share of it in the form of the chargeless, almost massless, particles known as neutrinos.

The 1% of a supernova's energy not emitted as neutrinos produces the spectacular visible explosion, and is deposited as a highly energetic SNR that expands into the surrounding gas, sweeping it into a dense shell. The expansion of this shell slows with increasing radius¹⁰ until it merges with the interstellar medium, maintaining that medium's turbulence. This process takes about a million years.

From the eight supernovae visible to the naked eye that have been recorded during the past two millennia¹¹, and from comparison with other galaxies, we infer that our Galaxy produces two or three supernovae per century. Accordingly, there should be thousands of SNRs. But we know of only 265, all through measurements of radio-frequency synchrotron radiation emitted by relativistic electrons produced by the supernova explosion¹. This number does not, therefore, include SNRs with no radio emission. Moreover, the observed range of radio luminosity of SNRs covers at least a factor of 100; why some SNRs are bright and others are not is not known. So the bias

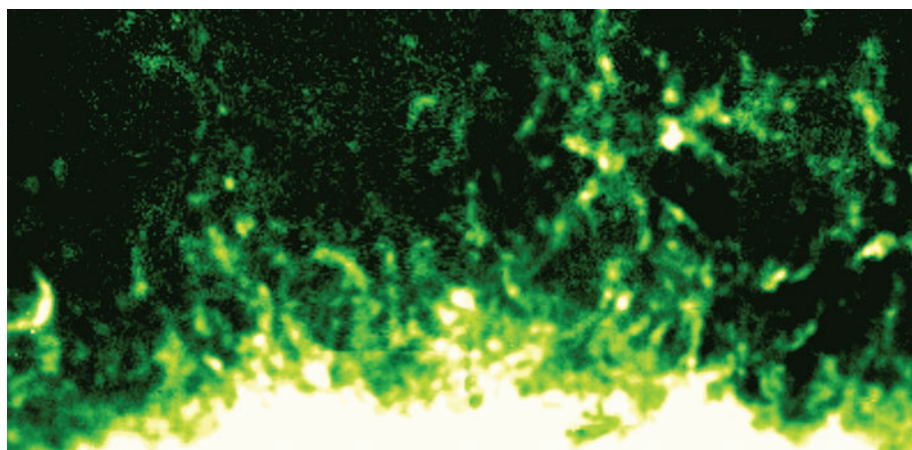


Figure 1 | Supernova supershell exposed. The most spectacular known supershell, produced by 100 or more supernovae, is revealed by this map¹² of emissions of the atomic hydrogen line at a wavelength of 21 cm, the tracer used by Koo and colleagues². The width of the image covers 20° of the sky; brighter yellow corresponds to higher and darker green to lower hydrogen density. The combined explosive energy has lifted the entire Galactic plane (white portion at the bottom) and sent shards of gas flying about around 6,500 light years into the Galactic halo, from which they will eventually return. (Figure courtesy of Y. Pidopryhora.)

of the current SNR sample towards the brighter, younger, radio-emitting supernovae is unknown, and the sample's statistical properties are correspondingly unreliable. A different technique for identifying supernova remnants is needed.

Enter Koo, Kang and Salter². When the expansion of an SNR slows down, its shell of swept-up gas turns into neutral atomic hydrogen (H I). The shell's velocity remains anomalous compared with that of the surrounding medium for some time, and, if the shell's angular diameter is large enough, it can be distinguished from ambient gas through a Doppler shift of the characteristic spectral emission line of neutral hydrogen at a wavelength of 21 cm. Using existing low-resolution H I surveys, Koo and colleagues² identified some 200 new SNR candidates. They mapped one, named FVW190.2+1.1, in detail using the Arecibo telescope on Puerto Rico. This SNR has a radius of some 290 light years, a velocity of expansion of 77 km s^{-1} , and an age of 340,000 years. SNRs as old as this lose all detectable radio emission, so the H I technique opens up an unknown and unsampled set of remnants. Compared with catalogued SNRs, FVW190.2+1.1 is situated unusually far (around 52,000 light years) from the centre of the Galaxy.

An intriguing aspect of this H I technique is its ability to see so-called supershells. Type II supernovae come from massive stars, which are formed in groups localized in space and time. These stars have fairly short, similar lifetimes, so multiple simultaneous supernovae can produce gigantic versions of SNRs. Two local examples of these supershells (or 'superbubbles'), the Orion–Eridanus superbubble and the North Polar Spur shell, dominate our sky, with each being tens of degrees in diameter. Several dozen more distant supershells have been catalogued, and the largest known supershell, at a distance from the Galactic

Centre of around 13,000 light years, has been mapped¹² from its emissions at 21 cm (Fig. 1). The production of this spectacular supershell required upwards of 100 supernovae; fortunately, this is consistent with its location in our Galaxy's star-forming hotbed.

Compared with the classical radio SNR identification, the H I technique emphasizes not just supershells, but also the more numerous older SNRs, and thus promises to remove the radio-selected statistical biases. The mapping of the whereabouts of interstellar gas with the 21-cm atomic hydrogen line is undergoing a renaissance as new multifield instrumentation at the Arecibo telescope¹³ and the Green Bank Telescope in West Virginia comes on stream. The future is bright for studying radio-dim SNRs.

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NEURODEGENERATIVE DISEASE

Pink, parkin and the brain

Leo Pallanck and J. Timothy Greenamyre

Dysfunctions in a number of cellular pathways can cause Parkinson's disease. Fruitflies with mutations in a protein called PINK1 show that there might be some unsuspected interplay between two such pathways.

Parkinson's disease was first described¹ in 1817, but our understanding of what causes the neurodegeneration that underlies its devastating symptoms is still rudimentary. Such poor understanding hinders the development of therapies, which currently don't seem to modify disease progression, even if they can mitigate for a time some of the movement difficulties that characterize this condition. In this issue, Clark *et al.* (page 1162)² and Park *et al.* (page 1157)³ provide clues to the basis of degeneration in Parkinson's disease by linking two causative mechanisms that were previously thought to be separate.

Most cases of typical 'sporadic' Parkinson's disease are believed to result from a lifetime of environmental exposures superimposed on an individual's genetic susceptibilities. However, a small fraction of cases (probably less than 10%) is caused by single-gene mutations. These provide insights into the cellular pathways involved in neurodegeneration⁴. For example, the products of two of the genes mutated in Parkinson's disease — parkin and ubiquitin carboxy-terminal hydrolase L1 — are components of the ubiquitin–proteasome system (UPS) that degrades damaged or misfolded proteins. Moreover, duplication or triplication of the normal alpha-synuclein gene causes Parkinson's disease, and overexpression of alpha-synuclein inhibits UPS function. Together, this evidence strongly implicates dysfunction of UPS in the development of Parkinson's disease.

Other single-gene mutations point to Parkinson's disease being associated with defects in mitochondria — the organelles that carry out respiration in the cell. Chemicals that inhibit the mitochondrial 'complex I' can reproduce many features of Parkinson's disease in experimental systems⁵. The mitochondrial hypothesis of Parkinson's disease was put on even firmer footing with the discoveries of causative mutations in the *DJ-1* and *PINK1* genes. The *DJ-1* protein has a role in the oxidative stress response; under oxidative conditions, some of the cellular *DJ-1* moves to mitochondria where its function remains to be elucidated. The *PINK1* protein is found primarily in mitochondria, and it is predicted to be a kinase (an enzyme that adds phosphate groups to other proteins), although its substrates are unknown⁶.

So, it looked as though there was the beginning of a nice, neat parcelling of two distinct mechanisms that cause Parkinson's disease: UPS dysfunction and mitochondrial impairment. Of course, things are rarely so simple,

and the first inkling that things were not quite as they seemed came from studies showing that overexpression of parkin in cultured cells delays toxin-induced mitochondrial dysfunction⁷ and that fruitflies that lack the *parkin* gene have prominent mitochondrial abnormalities in many tissues⁸. Mitochondrial defects were also observed in parkin-deficient mice and humans^{9,10}. Why would loss of this protein cause mitochondrial dysfunction?

Now Clark *et al.*² and Park *et al.*³ raise additional questions about what parkin is — or isn't — doing to cause Parkinson's disease, although the primary focus of these papers is the *PINK1* gene. Both papers show that fruitflies bearing mutations in the fly version of *PINK1* display degeneration of flight muscles and defective sperm formation. Using a combination of biochemical and imaging approaches, these researchers further report that mitochondrial defects accompany both of these abnormalities. Park *et al.*³ also report that the *PINK1*-mutant flies show loss of dopamine neurons — a type of neuron known to degenerate in Parkinson's disease — with an accompanying mitochondrial swelling. Although the specific tissues affected by loss of *PINK1* function differ in flies and humans, the fact that each of the tissues affected by *PINK1* mutations in the fruitfly has mitochondrial defects strongly suggests that the neurodegeneration in humans with *PINK1* mutations is also caused by mitochondrial dysfunction. This finding is interesting, but not unexpected.

The real surprise and importance of these papers is the finding that parkin seems to act downstream from *PINK1* in a common pathway that influences mitochondrial integrity. Indeed, the strikingly similar characteristics of the *PINK1*- and *parkin*-mutant fruitflies, including flight-muscle degeneration, sperm-formation defects and mitochondrial abnormalities, alone argue that these two genes act in a common pathway. Moreover, both papers show that overexpression of parkin compensates for a lack of *PINK1*, preventing the effects of the *PINK1* mutation. However, *PINK1* overexpression does not detectably influence the characteristics of the *parkin* mutants. Both papers also show that *PINK1*–*parkin* double mutants have symptoms that are indistinguishable from those seen in the single mutants. By contrast, the *DJ-1*-mutant fruitflies are quite different from *parkin* and *PINK1* mutants, suggesting that *DJ-1* influences different pathways. Interestingly, the similarities between the *PINK1*- and *parkin*-mutant fruit-

flies parallel a recent clinical study showing that in humans *PINK1* and *parkin* mutations can produce similar symptoms¹¹.

What is the nature of the pathway regulated by *PINK1* and parkin? The simplest interpretation of the data is that *PINK1* decreases parkin abundance by reducing the level of parkin messenger RNA or protein. Alternatively, *PINK1* may phosphorylate parkin and directly influence its activity. However, a problem with the latter model is that most data suggest that *PINK1* protein resides primarily in mitochondria and that parkin lies outside mitochondria. Nevertheless, it remains possible that the localization of either parkin or *PINK1* changes upon stress, and there are several reports, including that from Park *et al.*³, that have argued that at least some parkin associates with mitochondria.

Another model to explain the current findings is that *PINK1*-induced mitochondrial impairment leads to secondary dysfunction of the UPS, which can be ameliorated by overexpression of parkin. Parkin, a component of the UPS, is thought to act as a ubiquitin E3 ligase, an enzyme that directs proteins destined for destruction to the proteasome by tagging them with a ubiquitin group. In this regard, there is evidence that parkin mutations are associated with complex I defects¹⁰ and that complex I defects can inhibit the UPS in animal models of Parkinson's disease¹².

Finally, it remains possible that parkin has a functional role in mitochondria that does not involve its ubiquitin-ligase activity. Although there is substantial evidence *in vitro* that parkin can work as a ubiquitin ligase, few of the reported substrates of parkin have been validated *in vivo*. Furthermore, there is evidence that parkin can regulate the biogenesis of mitochondria¹³. Future experiments to delineate the *PINK1*–parkin pathway should clarify the mechanisms underlying neurodegeneration in Parkinson's disease and shed light on some very basic questions of mitochondrial biology. ■

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**Cover illustration**

Stem cells and their niche, along with differentiating germ cells, in the gonad of *Caenorhabditis elegans*. (Courtesy of J. Kimble.)

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STEM CELLS

More than 30 years ago, neurologist Oliver Sacks wrote of the Parkinson's drug L-dopa: "It is impossible to avoid the feeling that here, over and above all legitimate enthusiasms, there is this special enthusiasm, this mysticism, of a magical sort."

It is striking how aptly these words describe the current enchantment with stem cells. Now, 25 years after scientists first isolated mouse embryonic stem cells, it is possible to isolate and culture stem cells from embryos and adult tissues of many species, including humans. They are genetically no different from other cells in the body, so what combination of molecules confers their unique ability to self-renew and produce copious amounts of new cells, while maintaining the potential to form cell types of many lineages?

The identity of these molecules remains elusive, but, as the wellspring of growth, regeneration and healing, they are unlikely to remain hidden for long. And understanding their mechanisms may well transform medicine by enabling patient-specific therapies, in which a patient's adult somatic cells are reprogrammed to form genetically and immunologically matched stem cells. Such advances might one day make the use of human oocytes and embryos in stem-cell research a thing of the past. For the foreseeable future, however, the judicious use of human embryos and oocytes will be necessary for the very advances that will eventually make their use in stem-cell research obsolete.

Molecular reprogramming is just one facet of a field that is defined by its creative and interdisciplinary approach, as is embodied by the reviews in this Insight. No need for magical thinking here — the science is fascinating and superb. We hope these reviews convey some of the legitimate enthusiasm of stem-cell biology — well-tempered with reality.

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Natalie DeWitt, Senior Editor

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A glossary for stem-cell biology

Austin Smith¹

Stem-cell biology is in a phase of dynamic expansion and is forming connections with a broad range of basic and applied disciplines. The field is simultaneously exposed to public and political scrutiny. A common language in the stem-cell community is an important tool for coherent exposition to these diverse audiences, not least because certain terms in the stem-cell vocabulary are used differently in other fields.

Asymmetric division Generation of distinct fates in progeny from a single mitosis. Oriented division may position daughter cells in different microenvironments or intrinsic determinants may be segregated into only one daughter. Observed in some but not all stem cells and can occur in other types of progenitor cell.

Cancer cell of origin Precancerous cell that gives rise to a cancer stem cell. May be a mutated stem cell, or a committed progenitor that has acquired self-renewal capacity through mutation.

Cancer-initiating cell General term that encompasses both cancer cell of origin and cancer stem cell.

Cancer stem cell Self-renewing cell responsible for sustaining a cancer and for producing differentiated progeny that form the bulk of the cancer. Cancer stem cells identified in leukaemias and certain solid tumours are critical therapeutic targets.

Cell replacement therapy Reconstitution of tissue by functional incorporation of transplanted stem-cell progeny. Distinct from 'bystander' trophic, anti-inflammatory or immunomodulatory effects of introduced cells.

Clonal analysis Investigation of properties of single cells. Essential for formal demonstration of self-renewal and potency.

Commitment Engaging in a programme leading to differentiation. For a stem cell, this means exit from self-renewal.

Embryonic stem cell Pluripotent stem-cell lines derived from early embryos before formation of the tissue germ layers.

Founder/ancestor/precursor cell General terms for cell without self-renewal ability that contributes to tissue formation. In some cases they generate tissue stem cells.

Immortal strand The hypothesis of selective retention of parental DNA strands during asymmetric self-renewal. Potential mechanism to protect stem cells from the mutations associated with replication.

In vitro stem cell Self-renewal *ex vivo* in cells that do not overtly behave as stem cells *in vivo*. Occurs due to liberation from inductive commitment signals or by creation of a synthetic stem-cell state.

Label-retaining cell Candidate for adult tissue stem cell because of slow division rate and/or immortal strand retention. Interpret with caution.

Lineage priming Promiscuous expression in stem cells of genes associated with differentiation programmes.

Long-term reconstitution Lifelong renewal of tissue by transplanted cells. The definitive assay for haematopoietic, epidermal and spermatogonial stem cells. Transplantation assay may not be appropriate for all tissues.

Niche Cellular microenvironment providing support and stimuli necessary to sustain self-renewal.

Plasticity Unproven notion that tissue stem cells may broaden potency in response to physiological demands or insults.

Potency The range of commitment options available to a cell.

Totipotent Sufficient to form entire organism. Totipotency is seen in zygote and plant meristem cells; not demonstrated for any vertebrate stem cell.

Pluripotent Able to form all the body's cell lineages, including germ cells, and some or even all extraembryonic cell types. Example: embryonic stem cells.

Multipotent Can form multiple lineages that constitute an entire tissue or tissues. Example: haematopoietic stem cells.

Oligopotent Able to form two or more lineages within a tissue. Example: a neural stem cell that can create a subset of neurons in the brain.

Unipotent Forms a single lineage. Example: spermatogonial stem cells.

Progenitor cell Generic term for any dividing cell with the capacity to differentiate. Includes putative stem cells in which self-renewal has not yet been demonstrated.

Regenerative medicine Reconstruction of diseased or injured tissue by activation of endogenous cells or by cell transplantation.

Reprogramming Increase in potency. Occurs naturally in regenerative organisms (dedifferentiation). Induced experimentally in mammalian cells by nuclear transfer, cell fusion, genetic manipulation or *in vitro* culture.

Self-renewal Cycles of division that repeatedly generate at least one daughter equivalent to the mother cell with latent capacity for differentiation. This is the defining property of stem cells.

Stem cell A cell that can continuously produce unaltered daughters and also has the ability to produce daughter cells that have different, more restricted properties.

Stem-cell homeostasis Persistence of tissue stem-cell pool throughout life. Requires balancing symmetric self-renewal with differentiative divisions at the population level, or sustained asymmetric self-renewal.

Stemness Unproven notion that different stem cells are regulated by common genes and mechanisms.

Tissue stem cell Derived from, or resident in, a fetal or adult tissue, with potency limited to cells of that tissue. These cells sustain turnover and repair throughout life in some tissues.

Transit-amplifying cell Proliferative stem-cell progeny fated for differentiation. Initially may not be committed and may retain self-renewal.

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Nuclear reprogramming and pluripotency

Konrad Hochedlinger^{1,2} & Rudolf Jaenisch¹

The cloning of mammals from differentiated donor cells has refuted the old dogma that development is an irreversible process. It has demonstrated that the oocyte can reprogramme an adult nucleus into an embryonic state that can direct development of a new organism. The prospect of deriving patient-specific embryonic stem cells by nuclear transfer underscores the potential use of this technology in regenerative medicine. The future challenge will be to study alternatives to nuclear transfer in order to recapitulate reprogramming in a Petri dish without the use of oocytes.

Cells of a multicellular organism are functionally heterogeneous owing to the differential expression of genes. Historically, differential gene expression had been thought to involve the genetic elimination of those genes that were silenced and the retention of others that were expressed in a particular tissue. Cloning experiments in amphibians and mammals laid this idea to rest¹. They unequivocally demonstrated that adult cells are genetically equivalent to early embryonic cells, and that differential gene expression is the result of reversible epigenetic changes that are gradually imposed on the genome during development^{2–3}. The reversal of the differentiation state of a mature cell to one that is characteristic of the undifferentiated embryonic state is defined here as nuclear ‘reprogramming’. Reprogramming by nuclear transfer has been a unique tool for functionally testing nuclear potency, and for distinguishing between genetic and epigenetic alterations of various donor cells^{4–8}. The successful treatment of an animal model of disease by nuclear-transfer-derived embryonic stem (ES) cells (NT ES cells)⁹ and the prospect of deriving patient-specific human ES cells by nuclear cloning have underscored the potential use of this technology for custom-tailored cell therapy. However, although nuclear transfer remains the tool of choice for studying reprogramming at a functional level, alternative, more amenable approaches are needed for dissecting reprogramming at the cellular, molecular and biochemical levels.

This review discusses the different strategies that have been used to induce the conversion of a differentiated cell into an embryonic pluripotent state, including nuclear transfer, cellular fusion, the use of cell extracts and culture-induced reprogramming (Fig. 1). We critically discuss the criteria for assessing reprogramming at the functional and molecular levels using different approaches, and speculate on the molecular mediators that might facilitate reprogramming and the maintenance of pluripotency. In essence, reprogramming remains largely phenomenological; thus, future efforts should aim to dissect reprogramming at the molecular and biochemical levels.

Reprogramming by nuclear transfer

Most adult tissues contain a heterogeneous population of cells with a hierarchy of multipotent stem cells, progenitor cells and terminally differentiated cells. When ‘Dolly’ the sheep and other mammals were initially cloned from adult cells, the question remained of whether terminally differentiated cells are genetically totipotent. This was mainly owing to the lack of genetic markers that could unambiguously prove the differentiation state of the donor cell. The cloning of mice from mature lymphocytes that carried differentiation-associated immune-receptor

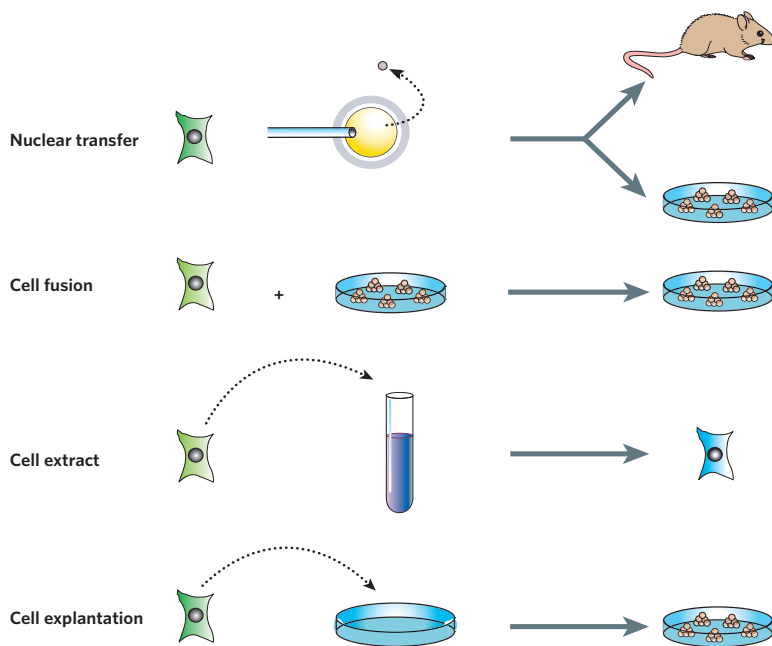
rearrangements⁵, and from genetically labelled postmitotic olfactory neurons^{6,8}, demonstrated that terminal differentiation does not restrict the potential of a nucleus to support development of an animal. In other words, the epigenetic changes that direct terminal differentiation and permanent exit from the cell cycle are reversible.

Cancer cells usually undergo both genetic and epigenetic changes that cause a block in terminal differentiation, and lead to uncontrolled growth. It has been unclear to what extent epigenetic changes contribute to tumorigenesis, and whether these changes are reversible. Nuclear transfer was used to address this question by cloning embryos from brain tumour⁷ and melanoma cells⁴. Chimaeric mice generated by injection of embryo-derived ES cells into blastocysts were normal⁴, indicating that the phenotype of some, but not all, of the tested cancer cells was largely due to epigenetic alterations that were reversible by nuclear transfer. Together, these observations underscored the importance of epigenetic modifications in regulating normal development, terminal differentiation and disease.

The generation of animals by nuclear transplantation is extremely inefficient, with most clones dying soon after implantation, and the few clones that survive beyond birth often being afflicted with severe abnormalities, such as obesity¹⁰ and premature death¹¹. Early experiments with amphibians demonstrated an inverse relationship between the age of the donor cell and clone survival¹². Therefore, an important question has been whether the state of donor-cell differentiation affects the efficiency of reprogramming in mammals. Reprogramming can be measured functionally by evaluating clone development at several different levels, including the rate of blastocyst formation, the fraction of cloned embryos surviving to birth or adulthood after implantation into the uterus, and the frequency with which pluripotent ES cells can be derived from cloned blastocysts explanted into culture (Fig. 2b). Pre-implantation development of reconstructed oocytes into blastocysts is particularly sensitive to experimental parameters, such as the cell-cycle stage and physical condition of the transferred nucleus, and is therefore not a useful measure of reprogramming efficiency. For example, the cell-cycle stage of the donor nucleus needs to be in synchrony with the metaphase-arrested oocyte. Because different donor-cell populations have different cell-cycle profiles, embryos cloned from asynchronous cell populations will undergo cleavage divisions at different efficiencies. Owing to this experimental variability during cleavage, measuring the fraction of live pups derived from reconstructed oocytes is also not a reliable criterion for quantifying reprogrammability. However, once an embryo has reached the blastocyst stage, it has a relatively consistent probability of developing into a mouse

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Strategies to induce epigenetic reprogramming



Experimental approach

Mechanistic insights

Reproductive cloning:
functional test for
reprogramming to totipotency

Allows epigenetic changes
(reversible) to be distinguished
from genetic changes
(irreversible)

Somatic cell nuclear transfer:
efficient derivation of
genetically matched ES cells
with normal potency

Limitations

Reproductive cloning is
very inefficient

There are abnormalities
at all stages of
development

Nuclear reprogramming of
somatic genome in hybrids
generated with pluripotent cells

Allows study of genetics of
reprogramming

Fusion rate is very low

Tetraploid cells are
generated

Exposure of somatic nuclei or
permeabilized cells to extracts
from oocytes or pluripotent cells

Allows biochemical and kinetic
analysis of reprogramming

No functional
reprogramming done

Explantation in culture selects
for pluripotent, reprogrammed
cells

Allows study of genetics of
reprogramming

May be limited to
germ line cells

Figure 1 | Different approaches for studying nuclear reprogramming. Illustration of the four major strategies used for studying nuclear reprogramming (left), and summary of the gained mechanistic insights and limitations of each strategy (right). First, nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte, which, upon transfer into a surrogate mother, can give rise to a clone (reproductive cloning), or, upon explantation in culture, can give rise to genetically matched ES cells (somatic cell nuclear transfer). Second, cell fusion of differentiated cells with pluripotent ES cells results in the generation of hybrids that show all features of pluripotent ES cells. Third, exposure of somatic cells or nuclei to cell extracts from oocytes or ES cells recapitulates early biochemical events of reprogramming without stably changing cell fate. Fourth, explantation of germ cells in culture selects for immortal cell lines that have regained pluripotency.

or giving rise to ES cells. Therefore, measuring the potential of a cloned blastocyst to generate a viable clone or ES cells provides more defined readouts for cloning efficiency than just development through cleavage stages. Figure 2 illustrates the variability in cloning efficiencies when comparing the different experimental readouts.

Previous cloning experiments with blastomeres of the cleavage-stage embryo suggested that pluripotent nuclei support clone development at high efficiency^{13,14}. Similarly, the cloning of mice from pluripotent ES cells^{15,16} has been shown to be more efficient than cloning from adult cells, such as fibroblast¹⁷, cumulus² or Sertoli cells¹⁸ (Fig. 2b; Table 1). Moreover, the derivation of ES cells from cloned blastocysts is significantly more efficient than the generation of mice from cloned blastocysts transferred into the uterus (Fig. 2b; Table 1). Explantation of cloned blastocysts in culture might, unlike fetal development, be under fewer time constraints and could select for the outgrowth of rare reprogrammed cells into stable ES-cell lines, thus resulting in an apparently higher reprogramming efficiency. Alternatively, reprogramming might not be restricted to the oocyte stage, but might continue in the inner cell mass of the blastocyst and, hence, give rise to NT ES cells at high efficiency. This observation might also explain why cloned mice from terminally differentiated neurons could only be generated using a 'two-step' procedure involving the initial derivation of NT ES cells, followed by the subsequent generation of cloned mice from NT ES-cell nuclei by a second round of nuclear transplantation⁶. The finding that mature natural-killer T (NKT) cells can give rise to cloned mice by a single round of nuclear transfer¹⁹ argues, however, that some terminally differentiated cells can generate clones following direct implantation into the uterus. Together, these results suggest the following conclusions: first, that mammalian nuclei, similar to those of amphibians¹², become more refractory to reprogramming with differentiation; second, that blastocyst formation and ES-cell derivation, in contrast to fetal development, are less restrained by genetic and

epigenetic abnormalities; and third, that ES-cell derivation from cloned blastocysts is significantly more efficient than the potential of cloned blastocysts to grow into live pups.

Whether the genomes of adult stem cells are similar to ES cells in that they are easier to reprogramme than the genomes of terminally differentiated cells is an open question. To address this, Billewicz *et al.* compared the cloning efficiencies of ES cells with that of cultured neural stem (NS) cells and differentiated fibroblasts²⁰. Cloned blastocysts produced from NS cells gave rise to ES cells about as efficiently as do cloned blastocysts derived from ES cells, and about two to three times more efficiently than do cloned blastocysts generated from fibroblast donors. In addition, fibroblasts that had been engineered to contain reduced levels of global DNA methylation were as efficient at being donors as were NS cells and ES cells, thus supporting the notion that the epigenetic state of donor cells affects the reprogrammability of cells, either through experimental manipulation or in the context of differentiation. However, this observation may not be true for all adult stem cells, because haematopoietic stem cells seem to be more efficient nuclear-transfer donors²¹ than differentiated B and T cells⁵, but less efficient donors than differentiated NKT cells¹⁹ of the same cell lineage (Table 1).

The developmental defects observed in reproductive cloning indicate faulty epigenetic reprogramming that should manifest itself in aberrant gene expression. Indeed, clones at various developmental stages, and even adult clones, show severe dysregulation of gene expression, with some genes being dysregulated in a donor-cell-dependent manner^{22–24}. The persistence of donor-cell-specific gene expression in clones indicates the retention of an 'epigenetic memory'^{22,25} of the donor nucleus, and might explain the observation that mice cloned from unrelated donor cells may suffer from different abnormalities^{10,11}. Faithful reprogramming of the somatic genome and complete elimination of the epigenetic memory of the donor nucleus seem to require passage through the germ line, because abnormalities are not seen in the offspring of clones¹⁰.

The notion that reproductive cloning fails to fully reprogramme a somatic nucleus raises the biologically and therapeutically relevant question of whether reprogramming is ever complete in ES cells derived from cloned blastocysts. Three lines of evidence suggest that this is, indeed, the case. First, NT ES-cell lines, once established, grow as immortal cell lines and produce pluripotent tumours when injected into immunocompromised mice²⁶. Second, analyses of the developmental potential of ES cells by tetraploid embryo complementation indicated that NT ES cells can give rise to entirely ES-cell-derived mice that appear normal at an expected frequency^{5,6,8,9}. Thirdly, global gene-expression profiling of NT ES-cell lines derived from different donor-cell types reveals transcriptomes that are indistinguishable from those of fertilization-derived ES-cell lines²⁷. The process of ES-cell derivation seems to rigorously select for immortal cells that have undergone, or continue to undergo, complete reprogramming to pluripotency, and, therefore, ES-cell lines derived by nuclear transfer are expected to have the same therapeutic potential as those derived from fertilized embryos.

An important question raised by these experiments is whether blastocysts and ES-cell lines can be derived by nuclear transfer from humans. Initial attempts to produce nuclear-transfer blastocysts from somatic donor cells have been unsuccessful²⁸ (Table 2). However, cloned blastocysts have been generated by injecting human donor nuclei into enucleated rabbit oocytes²⁹. These blastocysts failed to grow into stable ES-cell lines, which might have been owing to respiration defects caused by nuclear-mitochondrial incompatibility³⁰ — a phenomenon that has been observed in interspecies cell hybrids. An initial report on monkey cloning identified spindle abnormalities as a potential obstacle in primate somatic-cell nuclear transfer³¹, yet these difficulties were later overcome by modifying the nuclear transfer procedure³². Moreover, cloned monkeys have been generated from embryonic donor cells³³, suggesting that the cloning of primates works in principle. Although these and related questions regarding human nuclear transfer, such as the issue of the supply of human oocytes, clearly need to be resolved, alternative approaches to reprogramming deserve attention.

Reprogramming by cell fusion

Fusion between different cell types has been used to study the plasticity of the differentiated state³⁴ (Fig. 1). In most hybrids, the phenotype of the less-differentiated fusion partner is dominant over the phenotype of the more-differentiated fusion partner. Consistent with this, Miller and Ruddle showed, in 1976, that the fusion of pluripotent teratocarcinoma cells with primary thymocytes resulted in the formation of pluripotent hybrids that shared all their features with the parental embryonal carcinoma (EC) cells, including the potential to induce tumours³⁵. The dominance of pluripotent cells over differentiated cells has also been shown in cell hybrids made between somatic cells and murine embryonic germ (EG)^{36,37} and ES^{36,38} cells, and this reprogramming potential seems to be conserved in human ES cells^{39,40}.

A crucial question raised by these experiments was whether the chromosomes of the somatic cell had been reprogrammed to pluripotency, or whether they were simply retained as silent cargo. At the molecular level, the reactivation of the silent X chromosome in female lymphocyte-ES-cell hybrids³⁸, the demethylation and reactivation of genes essential for pluripotency^{38,39}, and the expression of genes representative of all three germ layers in teratomas produced from hybrids³⁶ supported the interpretation that the somatic chromosomes had undergone epigenetic reprogramming. To test reprogramming at a functional level, F9 EC cells that can normally only produce undifferentiated tumours were fused with thymocytes to score for an increase in the differentiation potency of tumours. The majority of hybrid cells gave rise to well-differentiated tumours, consistent with the notion that the thymocyte genome had been functionally reprogrammed to pluripotency by the EC cell⁴¹. However, independent fusion experiments between EC cells and differentiated cells came to the opposite conclusion⁴², and suggested that the differentiated phenotype of tumours might have been due to the loss or dilution of an amplified gene that blocked differentiation in EC cells, rather than the reprogramming of the somatic genome. So far, there has been no convincing functional

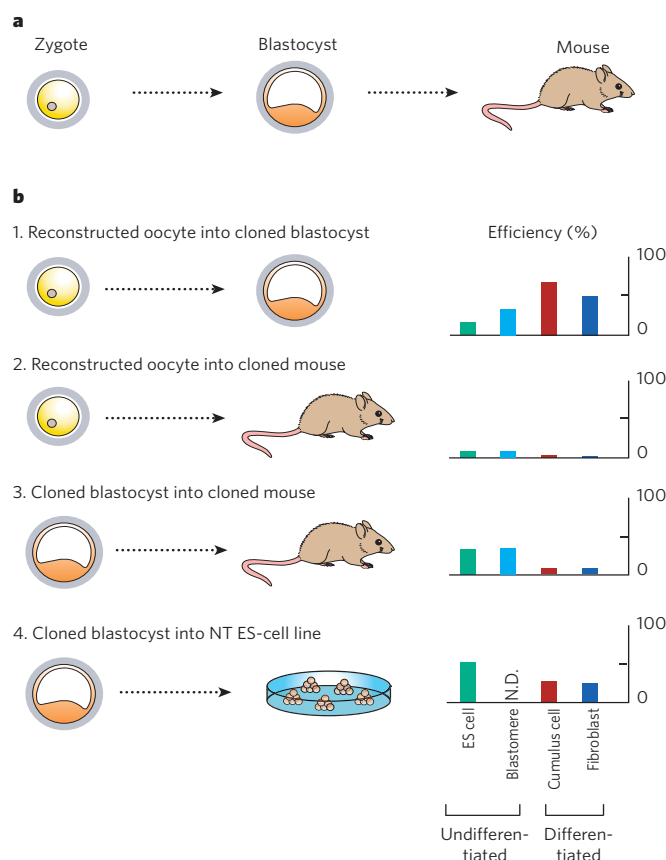


Figure 2 | Differentiation and cloning efficiency. **a**, Illustration of normal development of a mouse from pre-implantation development (zygote into blastocyst) to post-implantation development (blastocyst into mouse). **b**, The left side of the figure depicts four ways of assessing clone development by measuring pre-implantation development (1), a combination of pre-implantation and post-implantation development (2), post-implantation development (3) and the potency of a cloned blastocyst to give rise to ES cells (4). The right side of the figure shows the cloning efficiencies of four different donor-cell types for each category. ES cells and blastomeres are examples of undifferentiated cells, whereas cumulus cells and fibroblasts are examples of differentiated cells. Note the lack of correlation between donor-cell-differentiation state and cloning efficiency when considering the first two categories compared with the second two.

Table 1 | Cloning efficiencies of different donor cell types from different developmental stages

Donor cell	Blast into mouse (%) [*]	Blast into ES cell (%) [†]	References
Fertilized egg	60–80	25–68 [‡]	97
Blastomere	13–26 [‡]	ND	14
ES cell	11–23	50	15,16,61
EC cell	ND	50	61
NS cell	ND	64	20
Haematopoietic stem cell	0.7	ND	21
Sertoli cell	6 [‡]	27 [‡]	18,97
Cumulus	1–3 [‡]	9–19 [‡]	2,97
Fibroblast	1 [‡]	13–33 [‡]	17,97
Melanoma	ND	25	4
NKT cell	1–2 [‡]	6 [‡]	19
B/T cell	ND	7	5, unpublished data
Neuron	ND	6–28	6,8

^{*}The efficiency in terms of the percentage of mice obtained from the transfer of cloned blastocysts into surrogate mothers. [†]The percentage efficiency of deriving NT ES cells from cloned blastocysts explanted in culture. Note the high frequency of NT ES-cell derivation from cloned blastocysts compared with the frequency of mice produced from cloned blastocysts. [‡]The combined number of morulae and blastocysts that gave rise to ES cells or mice. ND, not determined.

evidence showing that the somatic donor nucleus has been fully reprogrammed and has re-gained the potential to sustain pluripotency or to direct differentiation in the absence of the ES-cell genome.

Two key questions arising from fusion experiments are whether the ES-cell nucleus or cytoplasm is required, and whether DNA replication is needed for reprogramming. The first question was addressed by separating the nuclear compartment (karyoblast) from the cytoplasmic compartment (cytoblast) of an ES cell; these elements were then individually fused with neuronal cells isolated from neurospheres⁴³. In hybrids produced with the ES-cell karyoblasts, reactivation of an *Oct4*–green fluorescent protein transgene was detected. By contrast, fusion of neurosphere cells with ES-cell cytoplasts gave no green fluorescent protein signal, suggesting that nuclear factors are essential for molecular reprogramming. This conclusion is consistent with cloning experiments in amphibians⁴⁴ and mice², which indicate that successful reprogramming depends on direct injection of nuclei into the germinal vesicle or into a metaphase oocyte, where nuclear factors are available in the cytoplasm.

The requirement for DNA replication for reprogramming is less clear. Although one ES cell–somatic cell fusion experiment suggested that replication is essential for reprogramming⁴³, nuclear transfer experiments indicated the presence of a replication-independent mechanism, possibly involving an active DNA demethylase⁴⁵. The different results might be due to biological differences in the cell types (ES cell versus oocyte) and/or technical differences in the assays (cell fusion versus nuclear transfer) used.

Reprogramming of somatic cells to pluripotency is a potentially attractive approach to generate customized cells for therapy without having to rely on nuclear transfer³⁹. However, for this approach to be viable, the ES-cell nucleus needs to be removed from the hybrid in order to generate diploid customized cells for transplantation therapy. If DNA replication and cell division are required for complete reprogramming it will be difficult, if not impossible, to selectively eliminate the entire set of ES-cell chromosomes from the hybrids.

Reprogramming by cell extracts

Using cell-free systems to study reprogramming is an attractive alternative to nuclear transfer or cell fusion (Fig. 1). Importantly, extracts can be used that might allow the purification of protein complexes involved in reprogramming. For example, exposure of permeabilized somatic frog cells to extracts prepared from *Xenopus* eggs showed that the somatic-cell-specific TATA-binding protein TBP is actively dissociated from chromatin through the activity of the ATP-dependent chromatin-remodelling factor ISWI⁴⁶, suggesting that major chromatin-remodelling complexes are involved in reprogramming. In a different set of experiments, human cells were exposed to *Xenopus* cell extracts and elevated *OCT4* transcript levels were detected following treatment⁴⁷. However, the latter study could not exclude the possibility that the detected *OCT4* signal came from a transcribed *OCT4* pseudogene or a *Xenopus Oct4* homologue, rather than from the reactivation of the endogenous *OCT4* locus. In addition, no stable reprogramming was seen in reversibly permeabilized somatic cells that were subsequently passaged in culture, suggesting that an intact oocyte might be required for functional de-differentiation to a pluripotent state.

Xenopus egg extracts have proved useful in solving an old puzzle in frog cloning. A recent study showed that efficient chromatin remodelling of differentiated nuclei depends on their exposure to mitotic egg extract, thereby facilitating embryonic DNA replication⁴⁸. Why frog cloning works better when using serial nuclear transfer has remained a mystery for decades⁴⁹. Serial nuclear transfer involves injecting the nucleus of a differentiated cell into an enucleated egg, allowing the cloned embryo to undergo a few cell divisions and then transferring the nucleus from one of the daughter cells into another enucleated egg. The biochemical approach now explains why embryos cloned serially from nuclei that have undergone previous rounds of embryonic DNA replication are more efficient donors than embryos cloned directly from adult cells.

In a conceptually simpler approach to induce reprogramming, permeabilized 293T cells were exposed for 1 h to extracts from EC cells⁵⁰ or

Table 2 | Facts and challenges in primate nuclear transfer

Human nuclear transfer Initial attempts by the company ACT unsuccessful²⁸; recent reports of successful derivation of human NT ES cells fraudulent⁹⁸; supply of human oocytes limiting for human nuclear transfer; possibility of generating ES-cell-derived oocytes^{99,100}

Human–rabbit xenotransplantation Successful generation of cloned blastocysts and inner-cell-mass outgrowths from the injection of human donor nuclei into enucleated rabbit oocytes²⁹; however, no stable ES-cell lines generated; possible respiration defects owing to mitochondrial incompatibility³⁰

Monkey cloning Successful with embryonic donor cells³³; spindle abnormalities observed in one report of somatic nuclear transfer clones³¹; abnormalities were alleviated by optimizing the nuclear transfer procedure³²

ACT, Advanced Cell Technology.

T-cell lines⁵¹, and *OCT4* or T-cell-receptor (TCR) expression, respectively, was detected by RNA and protein analyses. However, concluding that this indicated reprogramming of the somatic donor cells into other cell types is problematic. The presented data cannot exclude the possibility that gene products of the cells that were used to prepare the extracts were detected. A case in point is the reported TCR activation in reprogrammed fibroblasts. TCR expression requires functional genomic rearrangement of the TCR locus, which was not demonstrated in the reprogrammed fibroblasts, suggesting that the detected signal was due to transient uptake of TCR molecules from the extracts rather than activation of the endogenous TCR locus.

Culture-induced reprogramming

The approaches discussed so far require the exposure of somatic nuclei to nuclear/cytoplasmic factors of an oocyte or ES cell to elicit nuclear reprogramming. However, under certain physiological conditions, entire cells can de-differentiate or transdifferentiate into another cell fate (Fig. 1). Examples of cellular reprogramming include blastema formation during newt or zebrafish appendage regeneration, transdetermination of imaginal disc cells in flies, and reprogramming of germline cells in mammals and *Drosophila*. This review focuses only on cellular reprogramming in mammals, and the reader is referred to an excellent review in ref. 52 on the different forms of transdetermination and regeneration.

Teratocarcinoma cells were the first pluripotent cells discovered in adult mammals⁵³. Teratocarcinomas are members of a class of germ-cell tumours that are composed of a rare population of undifferentiated embryonic cells known as EC cells, as well as a range of differentiated cell types. These tumours have been experimentally shown to originate from primordial germ cells⁵³ (PGCs), which normally differentiate into oocytes or spermatozoa. The discovery of EC cells within teratocarcinomas prompted scientists to find the equivalent cells in normal embryos, leading to the isolation of pluripotent ES cells^{54,55} from pre-implantation-stage embryos, and pluripotent EG cells^{56,57} from isolated PGCs. Despite the phenotypic similarities of ES, EG and EC cells, functional and molecular differences exist that probably reflect their different cellular origins. For example, EG and EC cells show a more restricted developmental potential than ES cells, which presumably reflects their origin from PGCs that have lost genomic imprints^{58,59}. In addition, EC cells can form tumours when reintroduced into blastocysts⁶⁰ and this behaviour correlates with chromosomal changes that have accrued during tumour growth or *in vitro* culturing⁶¹. Nonetheless, germline contribution has been demonstrated for at least some EG^{58,62} and EC⁶³ cells, thus demonstrating their pluripotency.

The reprogramming of PGCs into EG and EC cells can be detected when comparing the developmental potencies of the cells of origin with their *in vitro* products (Fig. 3). For example, inner-cell-mass cells of the blastocyst and derivative ES cells are both pluripotent, and can give rise to all mouse cell types, including germ cells. Although the derivation of ES-cell lines^{54,55} from inner-cell-mass cells probably induces epigenetic changes that facilitate immortal growth, no differences in the developmental potentials have been observed before and after culturing of the cells. In contrast to inner-cell-mass cells and ES cells, PGCs do not contribute to tissues upon transfer into blastocysts⁶⁴. However, EG cells derived from explanted PGCs, and EC cells isolated from teratocarcinomas form

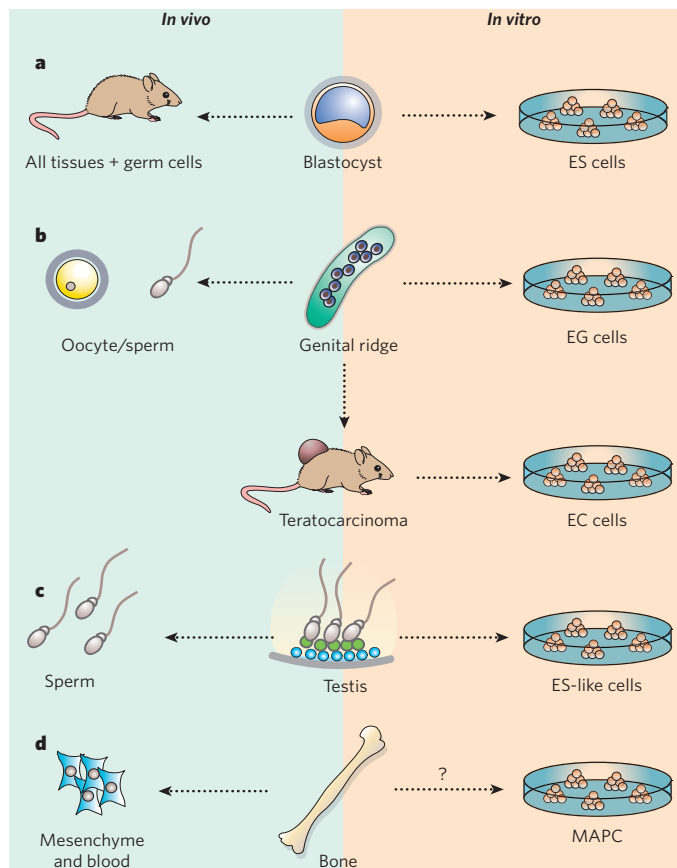


Figure 3 | Culture-induced reprogramming. Inner-cell-mass cells of the blastocyst give rise to all cell types of the body during normal development. **a**, Explantation of blastocysts in culture facilitates the outgrowth of immortal ES-cell lines but no change in developmental potential. **b**, PGCs of the genital ridge give rise to oocytes and spermatozoa *in vivo*, and facilitate the outgrowth of pluripotent EG cells *in vitro*. In teratocarcinomas derived from genital ridges, pluripotent EC cells are found that resemble EG cells. **c**, Spermatogonial stem cells from newborn or adult testes normally differentiate into spermatozoa, but occasionally give rise to pluripotent ES-like cells in culture. **d**, Bone-marrow-derived multipotent adult progenitor cells (MAPCs) might be the *in vitro* counterpart of mesenchymal or blood stem cells that have gained differentiation potential in culture.

tumours in mice with severe combined immunodeficiency disease and contribute to chimaeric animals after injection into host embryos^{58,62–64}. Differences between PGCs and derivative pluripotent cells are also seen when comparing the effect of deleting the *Oct4* gene on the phenotype of the cells. Deletion of *Oct4* in PGCs results in apoptosis⁶⁵, whereas loss of *Oct4* in ES cells causes differentiation⁶⁶. It might be that selective pressure imposed on the cells by transplantation to ectopic sites or by explantation in culture can relieve PGCs from certain restraints that normally control the terminal differentiation of germ cells. Loss of this control might facilitate proliferation and a gain of developmental potential. Convincing and reproducible evidence for the derivation of pluripotent cells has been confined to cells of the pre-implantation-stage embryo^{54,55,67} and the germ line^{56,57,68,69}. Germline cells, in contrast to somatic cells, undergo major epigenetic changes during their differentiation, which might render them more suitable for epigenetic reprogramming to pluripotency than somatic cells. It has been suggested, in fact, that all pluripotent cell lines characterized so far, including ES cells, are the product of germ-cell precursors⁷⁰. Therefore, an important issue has been whether pluripotent cells can be derived not only from the embryo but also from adults without previous manipulation of their nuclei.

Several reports have described the derivation of multipotent or pluripotent cell lines from adult tissues, including multipotent adult progenitor cells (MAPCs; Fig. 3) from adult bone marrow⁷¹ and unrestricted somatic

stem cells (USSCs) from human newborn umbilical cord blood⁷². These cells were shown to differentiate into cell types indicative of all three germ layers in culture and, when a single MAPC was injected into blastocysts, one extensive chimaera was reported⁷¹. This is surprising, as MAPCs divide infrequently, and to contribute to somatic tissues of the chimaera must successfully compete with the host epiblast cells that divide with a 6-h cell cycle⁷³. Although these results are intriguing, they await confirmation by independent laboratories. Also, it remains to be seen whether MAPCs and USSCs can functionally contribute to somatic tissues in animal models of disease or injury.

Recently, neonatal⁶⁸ and adult⁶⁹ testis cells were shown to give rise to ES-like cells when exposed to a specific combination of growth factors. ES-like cells expressed all the markers of pluripotent cells, formed teratomas after transplantation and gave rise to chimaeric animals that transmitted to the germ line. Thus, these cells represent the only clear example of the derivation of pluripotent cells from a normal neonatal or adult mammal, and might be useful for studying genetic diseases in different cell lineages. However, a potentially serious concern for any therapeutic application of these cells is the unbalanced genomic imprinting. Parental imprints are erased in PGCs and sequentially re-established in a male-specific or female-specific pattern during subsequent gametogenesis^{74–76}. Androgenetic ES cells are derived from two male gametes and have a male-specific pattern of imprinting, whereas parthenogenetic ES cells are derived from two female gametes and have a female-specific pattern of imprinting⁷⁷ (Table 3). Androgenetic ES cells are unable to contribute extensively to chimaeras, and fibroblasts derived in culture display an overtly transformed phenotype. By contrast, parthenogenetic ES cells show a broader differentiation potential, and fibroblasts rescued from chimaeric embryos undergo premature senescence⁷⁷. ES cells that have been genetically manipulated to be essentially ‘imprint-free’ develop into normal-appearing high-grade chimaeras upon injection into blastocysts⁷⁸. However, these chimaeras develop multiple types of cancer by 1 year of age⁷⁸. Spermatogonial stem cells, which originate from PGCs, must have undergone complete elimination of their imprints and probably have re-established some male-specific imprints. Thus, the ES-like cells derived from the spermatogonial stem cells are expected to have an unbalanced imprinting status that falls between that of ‘imprint-free’ ES cells⁷⁸ and androgenetic cells⁷⁷. This observation predicts that chimaeras derived from ES-like cells should be at least as prone to develop cancer as chimaeric mice derived from imprint-free ES cells (Table 3). Consequently, the therapeutic application of testis-derived ES-like cells might be problematic, because the unbalanced male-specific pattern of imprinted gene expression might inevitably result in tumorigenesis.

Molecular mediators of reprogramming and pluripotency

Cell-extract and nuclear transfer experiments have implicated chromatin-remodelling factors, DNA and histone modifications in the reprogramming of somatic nuclei. However, little is known about which genes are targeted by these modifications and are critical for reprogramming. There is a much better understanding of the genes that are important for sustaining pluripotency in ES cells and, as ES cells have reprogramming activity themselves, some of these genes might have a role in somatic-cell reprogramming.

The pluripotency of ES cells is maintained by a combination of extracellular and intracellular signals. Extracellular signals include generic signalling pathways, such as the signal transducer and activator of transcription 3 (STAT3), bone morphogenetic protein (BMP) and WNT cascades, whereas intrinsic signals comprise ES-cell-specific factors that execute the maintenance of pluripotency at the transcriptional level⁷⁹. So far, there has been no functional analysis of the effect of pluripotency-sustaining genes on cellular reprogramming. Indirect evidence, however, suggests that there is a correlation between aberrant reactivation of the pluripotency genes *Oct4*, *Nanog* and *Sox2* in blastocysts cloned from somatic cells and the abnormal development of the clones^{80–82}. By contrast, clones derived from ES, EG or EC cells that already express pluripotency genes showed faithful activation of OCT4 and exhibited an increased cloning efficiency^{61,81,82}.

Table 3 | Developmental and tumorigenic potential of pluripotent cells derived from donor cells with different imprinting states

Cells of origin	Imprinting status	Derivative pluripotent cell type	Developmental potential (chimaeras)	Tumorigenic potential (chimaeras)	References
Inner cell mass (from biparental embryo)	Biparental imprints	ES cells	Normal chimaeras	None	53–55
Imprint-free ES cells*	Erased imprints	ES cells	Overgrown chimaeras (no methylation imprints)	Multiple tumours	78, unpublished data
PGCs	Erased imprints	EG cells	Overgrown chimaeras	Not assessed	56,57,62,64,74–76
Inner cell mass (from uniparental embryo)	Male-only imprints	Androgenetic ES cells	Chimaeras abnormal, restricted contribution	Chimaera-derived fibroblasts transformed	77
	Female-only imprints	Parthenogenetic ES cells	Broad but uneven contribution to chimaeras	Fibroblasts undergo premature senescence	77
Spermatogonial stem cells (GS cells)	Erased and partly re-established male imprints	ES-like cells (mGS/maGS cells)	Normal chimaeras?	Not assessed	68,69

*Removal of all methylation imprints by sequential inactivation and reactivation of the DNA-maintenance methyltransferase 1 gene⁷⁸. GS cells, germline stem cells; mGS/maGS cells, multipotent/adult germline stem cells.

A key issue for current research is defining the downstream target genes of pluripotency genes. Genome-wide location analysis for OCT4, SOX2 and NANOG in human⁸³ and mouse⁸⁴ ES cells showed that these transcription factors collaborate to form specialized regulatory circuitry in ES cells. The target genes of these three regulators frequently encode other developmentally important transcription factor genes that are silent in the pluripotent undifferentiated cells, supporting the notion that inhibition of differentiation pathways is essential for the maintenance of pluripotency. Polycomb group (PcG) proteins are transcriptional repressors that function in maintaining cellular identity during metazoan development through epigenetic modification of chromatin structure⁸⁵. Recent evidence suggests that PcG proteins function to transcriptionally repress developmental genes in ES cells, the expression of which would otherwise promote differentiation^{86,87}. Many of the OCT4, SOX2 and NANOG target genes previously identified were also PcG targets, indicating that chromatin modifiers might act in concert with these three key pluripotency regulators to directly repress developmental pathways in ES cells. Interestingly, the chromatin conformation associated with many of these key developmental genes is composed of 'bivalent domains' consisting of both inhibitory histone H3 lysine 27 methylation marks and activating histone H3 lysine 4 methylation marks⁸⁸. These bivalent domains are lost in differentiated cells, suggesting that they play an important part in maintaining developmental plasticity of ES cells. Thus, the pluripotency factors OCT4, SOX2 and NANOG might act in concert with PcG proteins to silence key developmental regulators in the pluripotent state, while, for the same genes, positive epigenetic regulators are recruited and are poised to activate transcription upon differentiation. Once the transcriptional regulatory circuitry that confers pluripotency and self-renewal on ES cells is fully understood at the molecular level, it might be possible to reprogramme one cell type into another by affecting the activity of the key components of the transcriptional network.

Looking ahead

Will it be possible to fully reprogramme a somatic cell into an ES-like cell without exposure of the nucleus to the reprogramming factors of the oocyte? Will the genes and pathways essential for reprogramming be identified? Evidence from adult cells supports the notion that deletion or activation of single genes can facilitate the de-differentiation of mature cells into an immature state. For example, ectopic expression of CCAAT/enhancer-binding protein- α (C/EBP α) and C/EBP β in B cells facilitates their reprogramming into macrophages⁸⁹; similarly, loss of PAX5 (ref. 90) or ectopic expression of β -catenin⁹¹ in lymphoid progenitors endows them with multilineage differentiation potential. In the nervous system, oligodendrocyte precursors reprogramme into multipotent central nervous system-like stem cells when exposed to certain combinations of extracellular signalling factors⁹². Likewise, astrocytes lacking the *Ink4a/Arf* tumour-suppressor locus have the potential to reprogramme into NS cells in the presence of endothelial growth factor⁹³. Our laboratory has found that the ectopic activation of the pluripotency factor OCT4 in

adult mice results in the expansion of adult progenitor cells and tumour formation⁹⁴. Importantly, the tumours completely regress when OCT4 is turned off, owing to the instant differentiation of expanded progenitor cells. This finding suggests that adult progenitor cells remain responsive to this key embryonic transcription factor, and, thus, might identify adult progenitors as ideal targets for future reprogramming efforts. Some of the pluripotency-sustaining genes of ES cells have been identified through genetic screens⁹⁵; hence, it should be possible to devise gain-of-function and loss-of-function strategies to identify genes that facilitate reprogramming of somatic cells into ES-like cells. Lastly, it might be possible to further select for reprogrammed cells by exploiting the resistance of ES cells to loss of DNA methylation⁹⁶, which is a manipulation that is not tolerated by any other cell type. ■

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Asymmetric and symmetric stem-cell divisions in development and cancer

Sean J. Morrison¹ & Judith Kimble²

Much has been made of the idea that asymmetric cell division is a defining characteristic of stem cells that enables them to simultaneously perpetuate themselves (self-renew) and generate differentiated progeny. Yet many stem cells can divide symmetrically, particularly when they are expanding in number during development or after injury. Thus, asymmetric division is not necessary for stem-cell identity but rather is a tool that stem cells can use to maintain appropriate numbers of progeny. The facultative use of symmetric or asymmetric divisions by stem cells may be a key adaptation that is crucial for adult regenerative capacity.

Stem cells are defined by both their ability to make more stem cells, a property known as 'self-renewal', and their ability to produce cells that differentiate (Fig. 1a). One strategy by which stem cells can accomplish these two tasks is asymmetric cell division, whereby each stem cell divides to generate one daughter with a stem-cell fate (self-renewal) and one daughter that differentiates^{1–4} (Fig. 1b). Asymmetric division is a particularly attractive strategy because it manages both tasks with a single division; however, a disadvantage of this strategy is that it leaves stem cells unable to expand in number. This lack of flexibility is a problem, given that stem-cell numbers can increase markedly, both when stem-cell pools are first established during development^{5–7} and when they are regenerated after injury^{8–11}. Thus, asymmetric cell divisions cannot be the complete story. Stem cells must have additional self-renewal strategies that permit dynamic control of their numbers.

Stem cells can also use symmetric divisions to self-renew and to generate differentiated progeny. Symmetric divisions are defined as the generation of daughter cells that are destined to acquire the same fate. Although the idea that stem cells can divide symmetrically may seem counterintuitive, stem cells are defined by their 'potential' to generate more stem cells and differentiated daughters, rather than by their production of a stem cell and a differentiated daughter at each division. When viewed as a population, a pool of stem cells with equivalent developmental potential may produce only stem-cell daughters in some divisions and only differentiated daughters in others. In principle, stem cells can rely either completely on symmetric divisions (Fig. 1c) or on a combination of symmetric and asymmetric divisions (Fig. 1d). The evidence for symmetric stem-cell divisions is strong, both in model organisms such as *Caenorhabditis elegans* and *Drosophila*, and in vertebrates.

In this review, we explore the idea that most stem cells can divide by either asymmetric or symmetric modes of division, and that the balance between these two modes is controlled by developmental and environmental signals to produce appropriate numbers of stem cells and differentiated daughters. In this review, we define a cell division as asymmetric or symmetric according to the fates of its daughter cells. Available data, although often incomplete, suggest that most stem cells have the ability to switch between asymmetric and symmetric modes of division, and that the balance between these two modes of division is defective in some disease states.

Stem cells and asymmetric cell division

The roles of asymmetric cell division in stem-cell control, coupled with the mechanisms that regulate this process, have been extensively reviewed^{1–4}. In brief, two main types of mechanism govern asymmetric cell divisions. The first relies on the asymmetric partitioning of cell components that determine cell fate; we refer to such mechanisms as 'intrinsic'. The second involves the asymmetric placement of daughter cells relative to external cues; we refer to these mechanisms as 'extrinsic'.

Intrinsic mechanisms include regulated assembly of cell polarity factors (Fig. 2a) and regulated segregation of cell fate determinants (Fig. 2b). In situations in which the only difference between the daughter cells is their position relative to the stem-cell niche (Fig. 2c), the daughter cells may initially have equivalent developmental potential, but they may acquire different fates owing to exposure to varying external signals. In this way, the division is asymmetric with respect to the ultimate fate of the daughter cells even though the division is intrinsically symmetric, initially yielding two daughter cells with equivalent developmental potential. For many asymmetric divisions, the mitotic spindle is regulated so that its orientation is reproducible — a process that can be controlled by both extrinsic and intrinsic cues.

A classic example of an asymmetric division that is controlled by an intrinsic mechanism is provided by the *C. elegans* zygote, which divides asymmetrically to produce one larger blastomere fated to make ectoderm, and one smaller blastomere that produces mesoderm, endoderm and finally germ line in a series of asymmetric divisions³. Although not a traditional stem cell, this early embryonic lineage provides a model for asymmetric stem-cell divisions because each division produces one daughter cell that will produce only somatic cells and a second daughter cell that is capable of generating germ line. Furthermore, these embryonic divisions rely on mechanisms that are widely used by asymmetrically dividing stem cells and progenitors.

Asymmetric division of *C. elegans* zygotes requires asymmetric localization of the PAR-3, PAR-6 and atypical protein kinase C (PAR-aPKC) complex at the cortex (Fig. 2a; and reviewed in ref. 12). The asymmetrically localized PAR proteins in turn govern both mitotic spindle orientation and asymmetric segregation of cytoplasmic cell fate determinants, including riboprotein particles known as P granules and PIE-1, a transcriptional repressor required for germline fate^{12–16} (Fig. 2b).

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Asymmetric division of the *Drosophila* neuroblast is controlled by a closely related mechanism^{3,17}. Moreover, in *Drosophila* neuroblasts, an evolutionarily conserved cell fate determinant, Numb, is asymmetrically localized to daughter cells that are destined to differentiate¹⁸.

A classic example of an asymmetric division that is controlled by an extrinsic mechanism is provided by the *Drosophila* germline stem cell, which divides with a reproducible orientation to generate one daughter that remains in the stem-cell niche and retains stem-cell identity, and one daughter that is placed away from the niche and begins to differentiate^{4,19,20}. A stem-cell niche is defined as a 'microenvironment' that promotes stem-cell maintenance (refs 21, 22; see also page 1075). Cells that create stem-cell niches include cap cells in the *Drosophila* ovary¹⁹ and hub cells in the *Drosophila* testis^{23,24}. In the ovary, cap cells synthesize ligands called Decapentaplegic (DPP) and Glass bottom boat (GBB) that activate bone morphogenetic protein (BMP) signalling in germline stem cells, thereby repressing the gene *bag-of-marbles*^{25,26}, which encodes a protein that promotes differentiation²⁷. In the testis, hub cells synthesize a ligand called Unpaired that activates the JAK-STAT (Janus kinase and signal transducer and activator of transcription) signalling pathway in germline stem cells to prevent differentiation, presumably by controlling target genes that remain to be identified^{4,23,24}. Specialized junctions at the interface between the niche and germline stem cells anchor the stem cell to the niche^{28,29}. The mechanism controlling orientation of the mitotic spindle relies on centrosomal components in spermatogonial stem cells²⁹. More importantly for our discussion here, the orientation of these asymmetric stem-cell divisions controls the location of daughter cells and thus their access to extrinsic signals that regulate stem-cell identity.

It is important to note that asymmetric divisions can be governed by both intrinsic partitioning of fate regulators and asymmetric exposure to extrinsic cues. Sperm entry initiates asymmetry of the *C. elegans* zygote^{30,31}, and signalling from the neural epithelium orients divisions of *Drosophila* neuroblasts³² (Fig. 2c). Furthermore, Numb modifies the response to Notch signalling of the daughter cell that inherits it, indicating that cell fate determinants can function by altering the response to external cues³³. By contrast, asymmetric division of *Drosophila* germline stem cells does not seem to rely on partitioning of cell fate determinants. Although each germline stem cell is marked by a cytoplasmic organelle called the 'spectrosome', the function of this asymmetrically

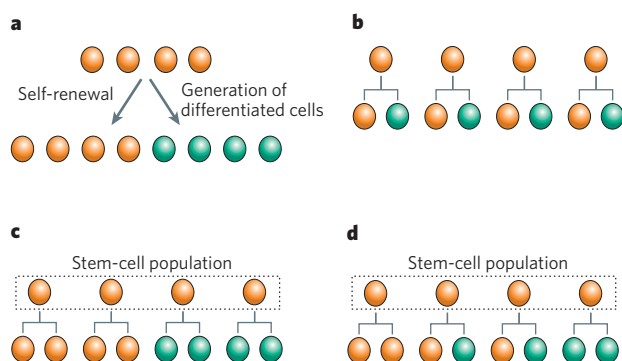


Figure 1 | Stem-cell strategies. **a**, Stem cells (orange) must accomplish the dual task of self-renewal and generation of differentiated cells (green). **b–d**, Possible stem-cell strategies that maintain a balance of stem cells and differentiated progeny. **b**, Asymmetric cell division: each stem cell generates one daughter stem cell and one daughter destined to differentiate. **c**, **d**, Population strategies. A population strategy provides dynamic control over the balance between stem cells and differentiated cells — a capacity that is necessary for repair after injury or disease. In this scheme, stem cells are defined by their 'potential' to generate both stem cells and differentiated daughters, rather than their actual production of a stem cell and a differentiated cell at each division. **c**, Symmetric cell division: each stem cell can divide symmetrically to generate either two daughter stem cells or two differentiated cells. **d**, Combination of cell divisions: each stem cell can divide either symmetrically or asymmetrically.

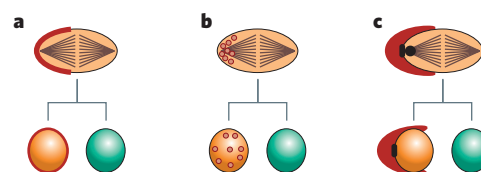


Figure 2 | Controls of asymmetric stem-cell division. Three simple mechanisms are shown, but others are plausible. For molecular details, see recent reviews^{1–4,12,17,31}. **a**, Asymmetric localization of cell polarity regulators (red) initiates the asymmetric division. Shown is asymmetric assembly of the PAR–aPKC complex at one end of the dividing cell. Stem cells are orange, differentiated cells are green. **b**, Cell fate determinants (red) can be segregated to the cytoplasm of one daughter cell, as shown here, or they can be associated with the membrane, centrosome or another cellular constituent that is differentially distributed to the daughters. **c**, Regulated orientation of the mitotic spindle retains only one daughter in the stem-cell niche (red), such that only that daughter cell has access to extrinsic signals necessary for maintaining stem-cell identity. This mechanism achieves an asymmetric outcome, even though the division itself is intrinsically symmetric. In an alternative but similar model, the daughter cell placed away from the niche is exposed to signals that induce differentiation.

distributed organelle remains uncertain. In addition, daughters of the germline stem-cell division seem to be equivalent in developmental potential, as we discuss below. Thus, these divisions seem to be intrinsically symmetric in terms of developmental potential, but seem to achieve an asymmetric outcome through the distinct positions of the daughter cells relative to the niche.

Some mammalian stem-cell divisions possess hallmarks of asymmetry and seem to be controlled by evolutionarily conserved mechanisms. An example is the division of neural progenitors. Undifferentiated neural progenitors in the developing rodent cortex distribute Numb asymmetrically to precursors destined for neurogenesis^{34–36}. The inhibition of Notch signalling by Numb is crucial for neurogenesis in flies, and it also seems to be involved in the regulation of mammalian asymmetric division^{33,37,38}. Numb is also asymmetrically distributed to progeny of cultured satellite muscle cells, where it promotes myogenic differentiation of one daughter cell³⁹. Thus, asymmetric segregation of Numb may be a common mode of control. A second example is the regulated orientation of mitotic spindles, which has been found in both mammalian basal epidermal progenitors⁷ and cortical ventricular zone neural progenitors⁴⁰. Indeed, spindle orientation relies on the cortical localization of the conserved PAR–aPKC complex⁷, a mechanism that also controls the asymmetric division of *Drosophila* neuroblasts^{41,42}. Thus, mammalian progenitors are likely to use some of the mechanisms of invertebrate progenitors to divide asymmetrically.

Symmetric divisions can expand stem-cell number

Symmetric stem-cell divisions have been observed during the development of both invertebrates and vertebrates. Symmetric stem-cell divisions are also common during wound healing and regeneration. A hallmark of all three processes is an increase in the number of stem cells. This increase cannot be explained by a strategy restricted to asymmetric cell division in which only one daughter cell maintains stem-cell identity.

A classic example of symmetric stem-cell division during development occurs in the *C. elegans* germ line. The larval nematode hatches from its eggshell with only two germline stem cells but, during subsequent larval development, these germ cells proliferate to produce roughly 2,000 descendants in the adult gonad, including one pool of undifferentiated germ cells and another pool of differentiating gametes^{5,43} (Fig. 3a). During larval development and in adults, *C. elegans* germline stem cells are maintained by signalling from a niche formed by the 'distal tip cell'⁵. In contrast to the *Drosophila* niches, which rely on BMP and JAK–STAT signalling^{23,24,44,45}, the distal tip cell niche uses Notch signalling to control *C. elegans* germline stem cells throughout development and in adults⁴³.

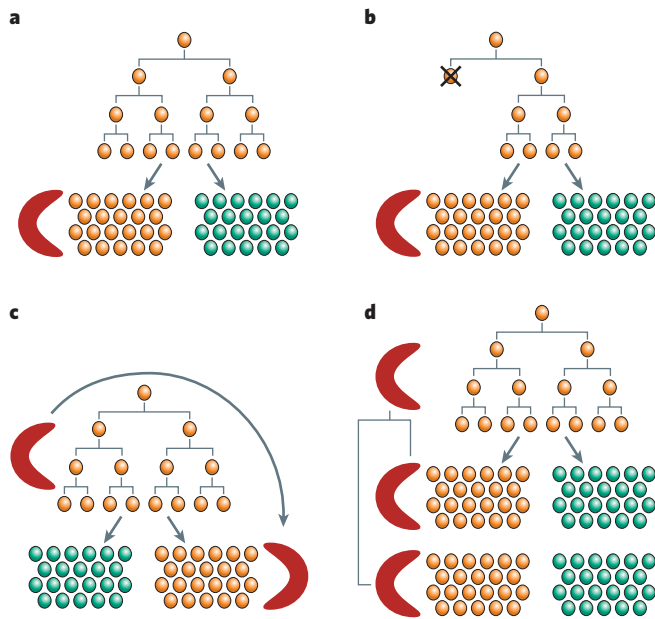


Figure 3 | Symmetric divisions in the developing *C. elegans* germ line.

a, *C. elegans* germline divisions during development are symmetric with respect to size and morphology of daughter cells, cleavage plane and position⁴⁶. Continued mitotic divisions rely on signalling from the stem-cell niche^{5,46,87}. Stem cells are orange; differentiated cells are green; the stem-cell niche is red. **b**, Elimination of one or more germ cells by laser ablation (marked with a cross) during early (shown) or later larval development does not affect the ability to generate pools of stem cells and differentiated cells⁵. Mitotic germ cells are therefore developmentally equivalent. **c**, Repositioning the niche induces germline stem cells at the new position⁵. **d**, Niche duplication results in duplication of the germline stem-cell pool. Niche duplication has been accomplished by alterations in either the cell-cycle machinery^{47,48,50} or regulators of niche specification^{49,51}.

Several lines of evidence show that *C. elegans* germ cells divide symmetrically during larval development. First, these divisions produce daughters of equal size and morphology, and they are variable with respect to both plane of cleavage and daughter cell position⁴⁶. Second, one or more germ cells can be removed by laser ablation without eliminating the capacity of the stem cells for both self-renewal and generation of gametes⁵ (Fig. 3b). Third, experimental repositioning of the stem-cell niche during early development — a time when all germ cells are proliferating — results in maintenance of the stem-cell fate by whatever germ cells happen to be located near the new niche position⁵ (Fig. 3c). Last, duplication of the niche results in duplication of germline stem-cell pools^{47–51} (Fig. 3d). Thus, during the expansion phase of germline development, *C. elegans* germ cells generate daughters with equivalent developmental potential, and these daughter cells ultimately acquire distinct fates depending on the position and number of niche cells. A similar phenomenon of symmetric germ cell divisions during larval development has recently been documented in *Drosophila*⁵².

Mammalian stem cells also seem to undergo largely symmetric divisions to expand stem-cell pools during embryonic or early fetal development. For example, mouse haematopoietic stem (HS) cells double in number every day during mid-gestation⁶, implying that a substantial fraction of these stem cells must undergo symmetric self-renewing divisions. However, direct imaging of these stem-cell divisions has not been possible. By contrast, it has been possible to image both the divisions of undifferentiated neural progenitors in cultured slices of the developing rodent cerebral cortex^{40,53,54} and cell divisions in the basal layer of the fetal epidermis⁷. During embryonic development of the cerebral cortex and epidermis, the pool of undifferentiated progenitors initially expands in number before significant amounts of differentiated cells are generated. During this expansion, cell divisions in the cortical ventricular zone generate two morphologically identical daughter cells

that seem to be undifferentiated and lie side by side in the ventricular zone where stem cells are located. Similarly, cell divisions in the fetal epidermis seem to be largely symmetric, generating morphologically equivalent undifferentiated cells in the plane of the basal layer of the epidermis where stem cells are present⁷. It remains formally possible, however, that morphologically and positionally equivalent progeny in locations known to contain stem cells could have different developmental potentials. Thus, without direct information on developmental potential or fate, inferences regarding symmetric versus asymmetric divisions of stem cells are based on incomplete criteria and should be considered provisional.

Symmetric divisions can persist into adulthood

Symmetric stem-cell divisions are common in developing tissues, but they can also be observed in adults, as exemplified by the adult *Drosophila* ovary. As described above, adult *Drosophila* germline stem cells normally divide asymmetrically⁵⁵ (Fig. 4a); however, female germline stem cells can be induced to divide symmetrically and to regenerate an additional stem cell after an experimental manipulation in which one stem cell is removed from the niche (Fig. 4b). Thus, adult *Drosophila* germline stem cells are regulated to divide asymmetrically or symmetrically.

Recent experiments further suggest that the daughters of *Drosophila* germline stem cells have equivalent developmental potential despite their distinct cellular morphologies (Fig. 4c). In these experiments, germline stem cells were induced to differentiate by altering specific regulators: in the ovary, the *bag-of-marbles* activator of differentiation was ectopically expressed by using a heat shock promoter⁵²; in the testis, the *stat92E* stem-cell activator was depleted by using a temperature-sensitive mutant⁵⁶. The former experiment also required ectopic expression of the DPP ligand in somatic ovarian cells. In both studies, germline stem cells located in the niche lost their stem-cell morphology and adopted cellular characteristics of a daughter fated to differentiate (Fig. 4c). When the activities of the regulators were reversed, however, the differentiating cells reverted to a stem-cell morphology and resumed a stem-cell fate.

The simplest explanation is that asymmetric divisions of *Drosophila* germline stem cells produce daughters of equivalent potential but place them in different positions with respect to signalling from the niche (Fig. 4d). These equivalent daughters then adopt distinct identities depending on the presence or absence of signalling from the niche (Fig. 4d). This idea could have been tested more directly, either by reversing the positions of the two daughter cells with respect to the niche to see whether their fates could be reversed, or by removing the stem cell by laser ablation to determine whether its differentiating sister could enter the niche and adopt the stem-cell fate. However, such physical manipulations are technically challenging in this system.

Symmetric stem-cell divisions are also common in the adult *C. elegans* germ line. Although individual germline stem cells in the adult gonad have not been identified by lineage tracing, a region of mitotically dividing germ cells called the 'mitotic region' is responsible for both self-renewal and replenishment of germ cells^{57,58} (Fig. 4e). Unlike *Drosophila* germline stem-cell divisions, which are reproducibly oriented with respect to the niche^{29,55}, *C. elegans* germ cells do not divide along any particular axis⁵⁸ (Fig. 4f, g). Indeed, about one-fifth of the germline stem-cell divisions maintain both daughters in the niche (Fig. 4e), whereas the other four-fifths place daughter cells in variable positions with respect to the niche (Fig. 4f).

A key unresolved issue is whether *C. elegans* germline stem cells divide asymmetrically with respect to their developmental potential. However, given the symmetric germline divisions in the early larval *C. elegans* germ line^{5,46}, and evidence that *Drosophila* germline stem-cell daughters are equivalent in developmental potential^{52,56}, it seems likely that adult *C. elegans* germline stem-cell divisions are also symmetric, and that differentiation results in daughters that become displaced from the niche. Consistent with this idea, molecular regulators promoting the differentiated state are expressed in germline daughter cells located outside the niche at a point about halfway through the mitotic region^{59,60} (Fig. 4e).

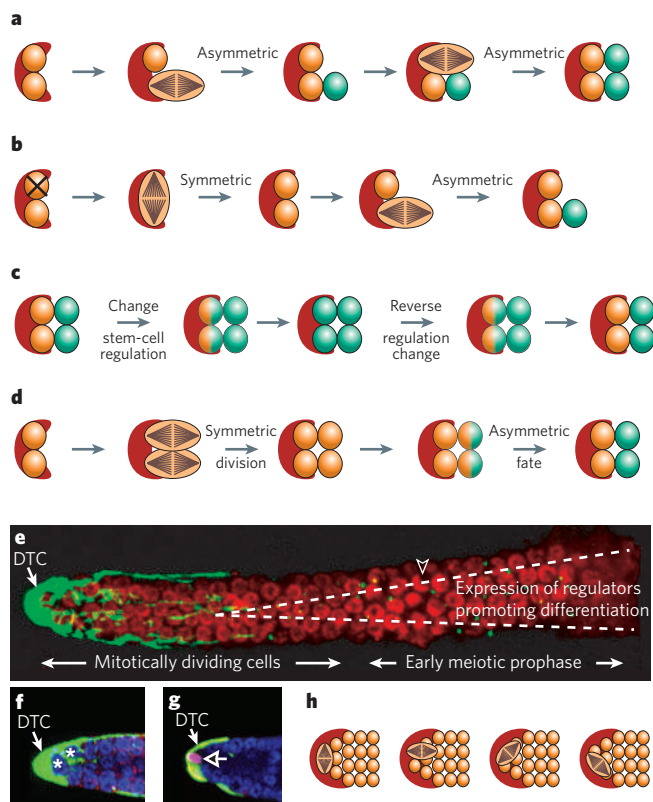


Figure 4 | Symmetric stem-cell divisions in the adult germ line. **a–d**, Adult *Drosophila* germline stem cells. **a**, Germline stem-cell divisions in the ovary are normally asymmetric. The niche (red) contains 2–3 germline stem cells (orange). Each divides with a mitotic spindle oriented toward the niche, retaining one daughter in the niche and placing the other outside the niche (green). **b**, Elimination of one germline stem cell (marked with a cross) leads to symmetric stem-cell division of the other germline stem cell¹⁹. **c**, Daughters of germline stem-cell division have equivalent developmental potential: germline stem cells in the niche can be induced to express cellular markers of differentiation (green) by manipulating stem-cell regulators^{52,56}. When this manipulation is reversed, the cells revert to a stem-cell fate. **d**, The experiments shown in **c** suggest that germline stem cells produce daughters with equivalent developmental potential, but the daughter outside the niche gradually changes into a differentiated state (graded orange to green). In this way, the oriented divisions yield daughters that acquire different fates despite initially having the same potential. **e–h**, Adult *C. elegans* germline stem cells. **e**, Germline stem cells in the adult gonad. The distal tip cell (DTC), shown expressing green fluorescent protein (green), provides a stem-cell niche for germ cells⁵ (red). The mitotic region includes ~225 germ cells; germline nuclei in early meiotic prophase are crescent-shaped (arrowhead). *gld-1* mRNA and protein products, which promote differentiation⁸⁸, are undetectable in germ cells close to the niche, but become detectable about half way through the mitotic region and are at higher concentrations (broken lines) as germ cells enter meiotic prophase⁵⁹. (Image adapted, with permission, from *WormBook*⁴³ and courtesy of S. Crittenden, Howard Hughes Medical Institute, Wisconsin, USA, and J.K.) **f**, The niche extends short processes that almost surround the distal-most germ cells (asterisks). These short processes may anchor the germ cells in the niche⁵⁸. (Image courtesy of S. Crittenden.) **g**, Approximately 20% of germ cells lying in the niche divide with an orientation that retains both daughters in the niche⁵⁸ (metaphase plate, pink). (Image courtesy of S. Crittenden.) **h**, The orientations of germline stem-cell mitotic spindles are not fixed⁵⁸.

Symmetric and asymmetric divisions of mammalian stem cells

Some mammalian stem cells seem to switch between symmetric and asymmetric cell divisions. For example, both neural and epidermal progenitors change from primarily symmetric divisions that expand stem-cell pools during embryonic development (Fig. 5a, c) to primarily asymmetric divisions that expand differentiated cell numbers in mid to late gestation (Fig. 5b). For these cells, divisions are classified as

symmetric or asymmetric depending on whether one or both daughter cells retain the position and morphology associated with stem cells.

As layers of differentiated cells arise in the forebrain, progenitors increasingly undergo apparently asymmetric divisions: one cell remains in the ventricular zone (where stem cells are located) and the other cell migrates into overlying layers of differentiated neurons^{40,53}. During formation of a stratified epidermis, asymmetric divisions begin to predominate at embryonic day 14.5 and lead to the generation of one cell that remains in the basal layer (where stem cells are located) and a second cell that migrates into a suprabasal layer of committed progenitors that are fated to undergo a limited number of symmetric divisions before differentiating^{7,61}. Thus, these mammalian stem cells seem to make a developmentally regulated transition from largely symmetric to predominantly asymmetric divisions during mid to late gestation. A caveat, however, is that mammalian stem cells cannot be distinguished from other progenitors on the basis of only morphology and position, so it remains possible that the frequency of asymmetric and symmetric divisions of stem cells differs from that observed in the overall pool of undifferentiated cells.

Only limited data are available on the modes of division used by adult mammalian stem cells *in vivo*. Adult mammalian stem cells are quiescent most of the time^{62,63}, and the rarity of adult stem-cell divisions makes them technically difficult to image. In most tissues, it is not known whether homeostasis is maintained by asymmetric divisions (Fig. 1b), or by a population strategy that uses symmetric divisions to balance stem cells and differentiated progeny (Fig. 1c, d). Nonetheless, evidence is starting to indicate that at least some adult stem cells divide asymmetrically under steady-state conditions to maintain population size (Fig. 5d). In the subventricular zone of the adult forebrain, for example, asymmetric divisions predominate under steady-state conditions, although some apparently symmetric divisions can be observed⁶⁴. Furthermore, clonal analyses based on retroviral marking of individual progenitors support the idea that undifferentiated neural progenitors divide asymmetrically^{65,66}. Homeostasis is also maintained in the adult oesophagus by apparently asymmetric cell divisions of progenitors in the basal layer⁶¹.

Although some adult stem cells seem to divide asymmetrically under steady-state conditions, they retain the capacity to divide symmetrically to restore stem-cell pools depleted by injury or disease (Fig. 5e), as has been observed in the nervous and haematopoietic systems. In the mouse forebrain, the frequency of subventricular zone cells capable of forming neural stem-cell colonies in culture is markedly reduced after infusion of an antimitotic drug. Neural stem cells begin dividing soon after the infusion, however, and within days they restore a normal frequency of cells that can form neural stem-cell colonies in culture¹¹. HS cells also divide symmetrically after injury, although it is not known whether they divide asymmetrically or symmetrically under steady-state conditions. In any event, when the haematopoietic system is decimated by chemotherapy, HS cells begin dividing and expand about tenfold in number to regenerate pools of both stem cells and differentiated cells^{8–10}. The common theme is that stem cells adopt a symmetric mode of division to regenerate depleted stem-cell pools after injury.

In mammals, symmetric divisions also increase in number after more physiological injuries. The death of rodent forebrain cells after stroke increases the rate of division among subventricular zone progenitors, including a rise in symmetric cell divisions that, in turn, leads to an increase in neurogenesis⁶⁴. These data are consistent with the above examples of stem cells that divide symmetrically to replace cells lost through injury. Subventricular zone progenitors, however, are heterogeneous and include both stem cells and other types of progenitor^{67,68}. So far there is no evidence for an increase in the absolute number of cells with stem-cell function in stroke; thus, it remains possible that the observed symmetric divisions occurred in transit-amplifying cells or restricted progenitors, rather than in stem cells. As new markers are discovered that rigorously identify mammalian stem cells *in vivo* and can distinguish these cells from intrinsically different populations of transit-amplifying cells, it will be possible to refine our understanding of mammalian stem-cell behaviour in the niche⁶⁹.

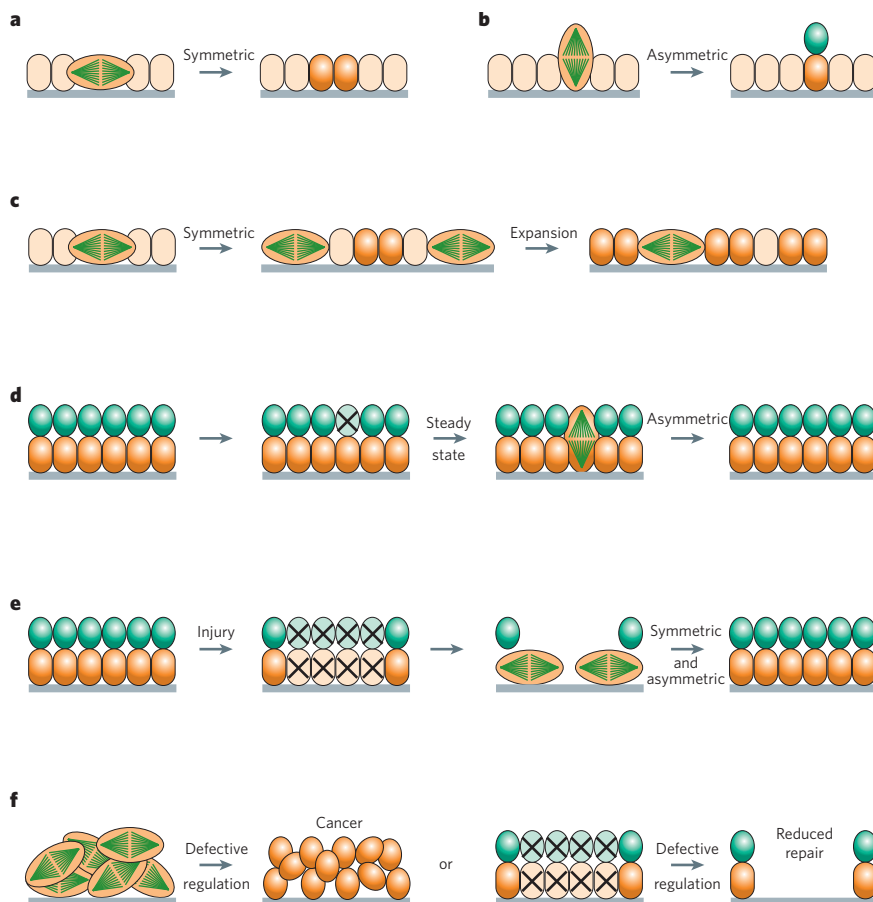


Figure 5 | Stem cells can facultatively use both symmetric and asymmetric divisions. **a**, Division in the plane of the epithelium generates two morphologically similar daughter cells that are both likely to be stem cells (orange). Grey line, basement membrane. **b**, Division perpendicular to the plane of the epithelium generates one stem cell and one differentiated daughter (green). Such asymmetric divisions by stem cells are thought to predominate during late fetal development and adulthood in the basal layer of epithelia^{7,61} and in the ventricular zone of the brain^{40,53}. Although spindle orientation seems to correlate with cell fate in this manner in various systems, it is not an obligate relationship because current data on progenitor identity and daughter cell fates are incomplete, and divisions in the plane of the epithelium can sometimes yield progenitors that acquire different fates⁵⁴. **c**, During development, symmetric divisions expand the stem-cell pool. **d**, In healthy adults, divisions perpendicular to the epithelial plane typically maintain normal numbers of stem cells and differentiated cells in the basal layer of epithelia and in the subventricular zone of the brain. **e**, In healthy adults, cells can be lost to injury (X). Symmetric divisions are proposed to regenerate additional stem cells, and asymmetric divisions to regenerate differentiated daughters. **f**, We speculate that defects in regulation of the switch between symmetric and asymmetric divisions can be deleterious. Left, a defect favouring symmetric divisions results in tumorigenesis. Right, a defect favouring asymmetric divisions results in decreased capacity for tissue repair. Both tumorigenesis and poor wound healing are typical of ageing animals, raising the question of whether defects in switch mechanisms accumulate with age.

Stem-cell divisions and cancer

The capacity for symmetric stem-cell self-renewal may confer developmental plasticity, increased growth and enhanced regenerative capacity; however, it may also confer an inherent risk of cancer. Normally, *Drosophila* neuroblasts divide asymmetrically³ as a result of the asymmetric localization of cortical cell polarity determinants (such as Partner of Inscuteable (PINS) and aPKC) and cell fate determinants (for example, Numb and Prospero), and regulated alignment of the mitotic spindle (Fig. 2). When the machinery that regulates asymmetric divisions is disrupted, however, these neuroblasts begin dividing symmetrically and form tumours^{42,70,71}.

Cell clones lacking PINS are tumorigenic^{42,71}, and double mutant cells lacking both PINS and Lethal giant larvae (LGL) generate a brain composed largely of symmetrically dividing and self-renewing neuroblasts⁴². Cell clones lacking the cell fate determinants Numb or Prospero are tumorigenic and can be propagated after transplantation into new hosts⁷¹. Moreover, these tumour cells have been shown to become aneuploid within 40 days of adopting a symmetric mode of division⁷¹. This finding indicates that invertebrate cells are capable of rapid neoplastic transformation. An intriguing possibility is that the capacity to divide symmetrically may be a prerequisite for neoplastic transformation and that cancer may reflect, at least in part, the capacity to adopt a symmetric mode of cell division.

The machinery that promotes asymmetric cell divisions has an evolutionarily conserved role in tumour suppression^{2,72}. The *adenomatous polyposis coli* (APC) gene is required for the asymmetric division of *Drosophila* spermatogonial stem cells²⁹ and is an important tumour suppressor in the mammalian intestinal epithelium^{73–75}. It is not known whether APC regulates asymmetric division by stem cells in the intestinal epithelium, but it is intriguing that, except for their unregulated proliferation, colorectal cancer cells have properties that are strikingly similar to those of intestinal epithelial stem cells⁷⁶. The human homologue of *Lgl*, *HUGL-1*,

is also frequently deleted in cancer^{77,78}, and deletion of the corresponding gene in mice leads to a loss of polarity and dysplasia in the central nervous system⁷⁹. Loss of Numb may be involved in the hyperactivation of Notch pathway signalling observed in breast cancers^{80,81}. Although these gene products could inhibit tumorigenesis through various mechanisms that are independent of their effects on cell polarity, the fact that these genes consistently function as tumour suppressors suggests that asymmetric division itself may protect against cancer.

Further evidence for the link between symmetric cell divisions and cancer is the observation that some gene products can both induce symmetric cell divisions and function as oncogenes in mammalian cells. One example is aPKC, the atypical protein kinase that normally localizes to the apical cortex of the neuroblast as part of the PAR–aPKC complex. Neural-specific expression of a constitutively active variant of aPKC causes a large increase in symmetrically dividing neuroblasts⁴². Consistent with this tumorigenic potential in *Drosophila*, aPKC has been also identified as an oncogene in human lung cancers^{82,83}. We speculate that asymmetric division may suppress carcinogenesis, in addition to its role in maintaining a balance between stem cells and differentiated progeny.

Symmetric modes of division may not only promote the expansion of stem-cell numbers, but also be permissive for secondary events leading to aneuploidy. Consistent with this possibility, the machinery that controls asymmetric division also regulates the orientation of mitotic spindles^{29,41,42}. A potential source of aneuploidy in symmetrically dividing fly neuroblasts is a defective centrosome — either duplicated or abnormal in shape — that presumably leads to errors in chromosome segregation⁷¹. The regulation of centrosome function by tumour suppressors is also important to avoid genomic instability in mammalian cells⁸⁴. Indeed, centrosomes and mitotic spindles seem to be tightly regulated in asymmetrically dividing cells to ensure that daughter cells adopt different fates. It is tempting to speculate that tightly regulated

centrosomes can also protect chromosomes from errors in segregation. If so, symmetric divisions might not only increase stem-cell numbers, but also increase the probability of aneuploidy and other secondary mutations by loosening the controls on mitotic spindles.

Perspective

The prolonged symmetric divisions of mammalian stem cells during early embryonic development generate large pools of stem cells and tissues that are capable of repair. Perhaps the ability to switch back and forth between symmetric and asymmetric modes of division, depending on developmental and environmental cues, is a key adaptation that increases the capacity for repair and facilitates longer lifespan. A potential cost of the increased use of symmetric divisions by stem cells may be a higher incidence of cancer, particularly given circumstantial evidence that cancers frequently arise from the transformation of somatic stem cells^{85,86}. Moreover, if tumour growth and progression are driven by cancer stem cells^{85,86}, then this process may remain biologically dependent on modes of division that permit the geometric expansion of stem cells.

The idea that symmetric divisions are required for neoplastic proliferation remains hypothetical, but raises the possibility that studies of the asymmetric division machinery could identify important new tumour-suppressor mechanisms. A key issue for the future is how stem cells are regulated to switch between asymmetric and symmetric divisions. A molecular understanding of this regulatory switch is not only relevant to basic stem-cell biology, but also has tremendous clinical importance for controlling stem cells therapeutically.

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The stem-cell niche as an entity of action

David T. Scadden¹

Stem-cell populations are established in 'niches' — specific anatomic locations that regulate how they participate in tissue generation, maintenance and repair. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation. It constitutes a basic unit of tissue physiology, integrating signals that mediate the balanced response of stem cells to the needs of organisms. Yet the niche may also induce pathologies by imposing aberrant function on stem cells or other targets. The interplay between stem cells and their niche creates the dynamic system necessary for sustaining tissues, and for the ultimate design of stem-cell therapeutics.

In architecture, the word niche refers to a recess, and in ecology it refers to a habitat where an organism can reside and reproduce; in French, it refers to a dog-house. So, the grand position of the stem cell in popular concepts of science is appropriately humbled by the cells dwelling in a place where they might awaken with fleas. However, the niche does provide both conceptual and physical depth to the stem cell. Stem cells are, after all, defined by their function in complex multidimensional environments over time. Stem cells are by themselves of limited interest, but the integration of stem cells and their descendants into tissues is the basis for higher forms of life. Therefore, the niche is as critical as stem-cell-autonomous functions in shaping our understanding of basic stem-cell biology, and how it contributes to health and disease.

Although a place of residence would fulfil the architectural conception of the niche, it is inadequate in regard to stem cells. The niche in cell biology is more appropriately considered along ecologic lines, and requires a sustaining function. The simple location of stem cells is not sufficient to define a niche. The niche must have both anatomic and functional dimensions, specifically enabling stem cells to reproduce or self-renew. This becomes particularly important as new sites of stem-cell localization are defined.

Adult or somatic stem cells generally have limited function without the niche. A case in point is the haematopoietic stem (HS) cell, which regenerates the entire blood and immune system, and makes copies of itself after limit-dilution transplantation. HS cells circulate freely, but seem to have little function outside specific anatomic locations. It is specific cues from specific sites that allow stem cells to persist, and to change in number and fate. Importantly, it is also the niche that provides the modulation in stem-cell function needed under conditions of physiologic challenge. It is this dynamic capability that makes the 'niche' concept particularly important and central to the realization of regenerative medicine. It is the ability of the niche to impose functions on stem cells that makes the concept important in disease. We are now at a time when descriptive studies are giving way to those of physiology. This review attempts to integrate the issues of niche components, signals and modification, and determinism imposed by the niche to portray the dynamism inherent in niche biology.

Niche elements reconsidered: the role of extracellular matrix

The concept of a niche as a specialized microenvironment housing stem cells was first proposed by Schofield almost 30 years ago in reference to mammalian haematology¹, although experimental evidence was first provided by invertebrate models. In the gonads of *Drosophila melanogaster*

and *Caenorhabditis elegans*, the germ stem cells reside at the distal end of a tapered structure, and have been shown to depend upon interactions with somatic cells at the end of that structure to maintain stem-cell features^{2–4}. The notion that heterologous cell types compose the niche was well supported by this early work and has led to emphasis on defining similar cell-based niche components in mammalian tissues. This has yielded identification of the osteoblast in the bone marrow, and the endothelium in the brain, and possibly in the bone marrow^{5–8}. Whether there is a necessity for another cell type — other than the stem cell — to be present for a niche to function, however, has recently been brought into question.

Two reports have demonstrated that the posterior mid-gut populations of cells in *Drosophila* have the capacity to produce daughter cells of at least two types: enterocytes and enteroendocrine cells^{9,10}. These cells seem to be able to self-renew and provide a long-lived ability to generate more mature offspring in a clonal manner. Notably, however, these cells do not appear to be in direct contact with a heterologous cell type. They do have focal localization of the β -catenin paralogue, Armadillo, at the interface between the stem cell and its descendant daughter cell (the enteroblast), but this is distinct from the cells of different lineages composing most previously defined niches. The stem cells in this case sit on a basement membrane that divides them from surrounding muscle cells. It is suggested that the basement membrane itself might participate in the specialized microenvironment, possibly providing an opportunity for shifting location of the stem cell, sliding along a continuum of the intestine. These data challenge the expectation that heterologous cells must necessarily be within the niche, and suggest that some stem cells might have a niche composed of extracellular matrix and other non-cellular constituents that could regulate their control (Fig. 1).

The concept of extracellular matrix regulating primitive cells is longstanding and at least three examples now exist in mammalian stem-cell systems. The first is in the skin, where β -1 integrins are known to be differentially expressed on primitive cells and to participate in constrained localization of a stem-cell population through presumed interaction with matrix glycoprotein ligands^{11,12}. Second, in the nervous system, absence of tenascin C alters neural stem-cell number and function in the subventricular zone¹³. Tenascin C seems to modulate stem-cell sensitivity to fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 4 (BMP4), resulting in increased stem-cell propensity to generate glial offspring. In the haematopoietic system, tenascin C deletion has also been shown to affect primitive cell populations, raising the possibility that it participates in several stem-cell niches¹⁴.

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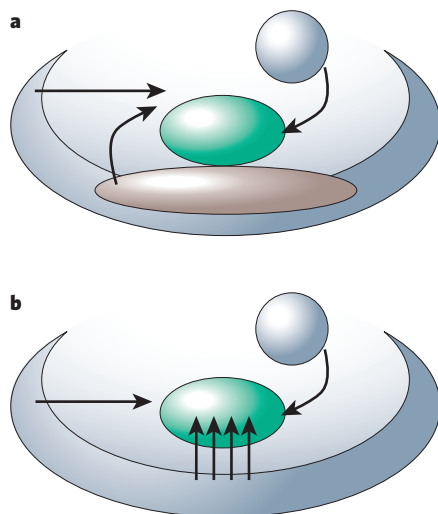


Figure 1 | Refining elements necessary for an adult stem-cell niche. **a**, Early studies provided evidence that heterologous cell types created a three-dimensional structure in which stem cells reside. **b**, Recent data raises the possibility that a regulatory microenvironment might include stem cells simply resident on the basement membrane with homologous cell–cell interactions. Stem cells are shown in deep green and more mature offspring are represented by a lighter shade.

Third, another matrix protein, the Arg–Gly–Asp (RGD)-containing sialoprotein, osteopontin (OPN), has now been demonstrated to contribute to HS-cell regulation^{15,16}. Implicated previously in tumour metastasis and cell-mediated immunity^{17,18}, OPN interacts with several receptors known to be on HS cells, CD44, and $\alpha 4$ and $\alpha 5\beta 1$ integrins^{19,20}. It is produced by multiple cell types, among them osteoblasts that contribute to the HS-cell niche. Notably, OPN production can vary markedly, particularly with osteoblast activation. Animals deficient in OPN have an increased HS-cell number, owing to a mechanism that is still debated but is microenvironment dependent^{15,16}. Without OPN, superphysiologic stem-cell expansion occurred under stimulatory conditions¹⁵. Therefore, OPN seems to serve as a constraint on HS-cell number, limiting the number of stem cells under homeostatic conditions or with stimulation. Taking these three scenarios together, matrix components provide localizing niche elements that can contribute stimulatory, or impose inhibitory, influences on the stem-cell pool. They, like other stimuli in the niche, balance opposing possibilities.

Paracrine factors and niche structure

Cells and extracellular-matrix components in the stem-cell niche are relatively predictable, although the complexity and integration of these elements is far from understood. Soluble mediators of cellular response would also be expected and a number have been defined that are important paracrine regulators of stem-cell function. Within the *Drosophila* testis, Unpaired (UPD) is secreted by niche (hub) cells and induces stem-cell self-renewal via Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling^{21,22}. In the *Drosophila* ovary, Decapentaplegic (DPP) is secreted after proteolytic cleavage and, along with another BMP analogue, Glass bottom boat (GBB), is produced by niche (cap) cells^{23,24}. These stimulate SMAD signalling in the germ stem cells and result in suppression of differentiation, maintaining the self-renewing stem-cell state²⁵. Wingless-related proteins (WNTs) and their antagonists, soluble Notch modulators, FGFs and Hedgehog (HH) also contribute input in a paracrine manner with varying capacity to induce proliferation or impair differentiation. HH, however, has an additional intriguing function: it is secreted by cap cells and mediates proliferative effects not just on germ stem cells, but also on surrounding somatic cells composing the niche itself in the *Drosophila* ovary^{26,27}. This effect on niche composition is a common theme of HH extending to mammals.

HH family members have a role in topographic organization and formation of niches in development. Sonic hedgehog (SHH) is essential for hair-follicle formation, and distinguishes the localization of specific subsets of epidermal stem cells to either the follicular niche or the interfollicular epidermis^{28,29}. In addition, SHH participates in specific spatially restricted events within established niches. Hair represents a uniquely dynamic physical association of elements within a niche, owing to the change in distance during the hair cycle of the dermal papilla from the bulge region where at least some stem cells reside. Expression of SHH is spatially and temporally restricted, and seems to modulate new hair formation³⁰, which is a function amenable to small-molecule manipulation³¹. In the developing intestine, SHH and Indian hedgehog contribute to architectural patterning, playing a key part in assembly, dimensions and regularity of the crypts where stem cells reside³². Therefore, HH contributions to stem-cell control are morphogenic, creating a niche, and regulating stem-cell fate within that niche.

Maintaining physical organization of the established niche is probably an active and important process; however, the signals governing it are not well defined. Indirect evidence suggests that ephrins, important mediators of structural boundaries in neural and vascular tissue, might participate in at least one stem-cell system. Graduated expression of ephrin B1 and, reciprocally, its transmembrane tyrosine kinase receptors, EphB2 and EphB3, has been elegantly shown to organize mouse intestinal epithelial cells³³. Disrupted expression of the receptors leads to aberrant organization of crypt and villus cells, including cells at the interposition between them, which are defined as stem cells. The altered topographic orientation of primitive and maturing cells results in polyploid outgrowths. Therefore, it will be important to discern molecular events sustaining architectural organization of the niche, particularly in considering how niche elements might participate in dysregulated growth in tumours.

Metabolism as an underexplored variable

Cells, matrix glycoproteins and the three-dimensional spaces they form provide ultrastructure for a stem-cell niche. The contact between these elements allows molecular interactions that are critical for regulating stem-cell function. Secreted proteins offer a paracrine measure of control, but non-protein components of the local microenvironment also affect stem-cell function. One such participant was examined for the haematopoietic niche based on the unusual mineral composition of the bone adjacent to which at least a portion of the stem cells reside. It was proposed that because the endosteal surface is the location of bone remodelling, and osteoblasts and osteoclasts are often co-localized, the known high ionic calcium concentrations that exist near active osteoclasts might influence stem-cell function. Osteoblasts express receptor-activator of nuclear factor- κ B ligand (RANKL), which is essential for osteoclast differentiation and, thus, the proximity of osteoblasts and osteoclasts. At the site of active bone remodelling, ionic concentrations have been observed that dramatically exceed serum levels and vary with osteoclast activity. Cells sense extracellular ionic calcium through the seven membrane-spanning calcium-sensing receptor (CaR). This G-protein-coupled receptor was found to be expressed on a stem-cell-enriched population of bone-marrow haematopoietic cells, as well as select subsets of mature haematopoietic cells³⁴. Stem cells from mice genetically engineered to be CaR deficient had a markedly abnormal ability to engraft the bone marrow. They were functionally normal in the fetal liver and spleen, but were unable to engage with or be retained at the endosteal niche. Simple molecules, including inorganic ions, might contribute to niche function.

Metabolic products, such as calcium, which influence stem-cell behaviour in the niche are likely to represent a dimension of stem-cell control that allows cells to respond to varying conditions of tissue state. For example, oxidative stress markedly affects stem-cell function. Under conditions in which 'ataxia telangiectasia mutated' (ATM) deficiency was induced, reactive oxygen species (ROS) accumulated in HS cells in the bone marrow³⁵. This was associated with increased

levels of the cyclin-dependent kinase inhibitor associated with senescence, p16INK4a, and with evidence of markedly decreased stem-cell function. Modifying ROS with anti-oxidants reduced p16INK4a and rescued stem-cell function. Challenges of oxidative stress are therefore likely to be important in altering stem-cell fate. The changing local metabolic conditions reflecting tissue state might thereby provide real-time information to modulate stem-cell function. Metabolic sensing is a likely mechanism by which stem cells gauge the 'needs' of a tissue or organism (Fig. 2). This requires further detailed analysis.

What changes in a niche?

Experimental examination of systemic inputs to the niche might include measuring stem-cell number or proliferation. However, the haematopoietic system provides an additional feature, stem-cell trafficking, which can be readily quantitated, making it attractive as an experimental model. Stem-cell entry and exit from the niche are important components of haematopoietic niche function that are essential in development and persist in the adult.

Blood formation in early development occurs in distinct extra-embryonic and embryonic sites. The yolk sac, aorto-gonadomesonephros (AGM), placenta and fetal liver participate in embryonic and definitive haematopoiesis in temporal sequence. Early haematopoietic-cell production occurs with apparent independence in the yolk sac and AGM regions. When the fetal liver becomes a source of blood-cell production, this is thought to occur by virtue of cells migrating from the AGM or placenta. Similarly, as haematopoiesis transitions to the bone marrow, migration rather than *de novo* generation is thought to be critical. This is best exemplified by mice made genetically deficient in CaR, or in the chemokine CXCL12 or its cognate receptor CXCR4, which have normal production of fetal liver HS cells, but fail to transition to haematopoiesis in the marrow space^{34,36,37}. These phenotypes are due to HS cells failing to move to the marrow niche.

The migratory nature of HS cells is therefore fundamental to their developmental programme and remains ongoing in adulthood. HS cells are found in the blood even under homeostatic conditions in mice and, from what can be estimated on the basis of imperfect measures, also in humans. These numbers can be dramatically modified on the basis of stimulation by several cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF and

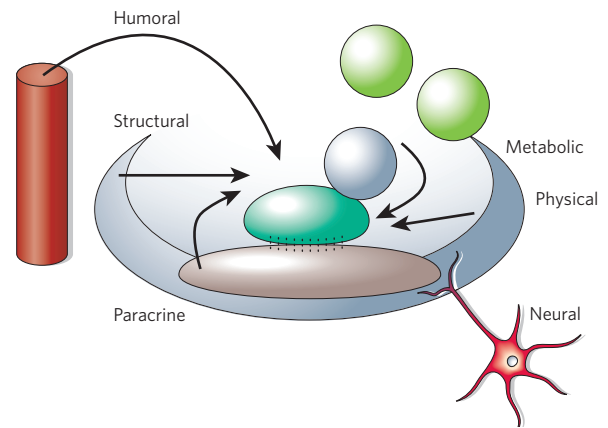


Figure 3 | Inputs feeding back on stem-cell function in the niche. Elements of the local environment that participate in regulating the system of a stem cell in its tissue state are depicted. These include the constraints of the architectural space, physical engagement of the cell membrane with tethering molecules on neighbouring cells or surfaces, signalling interactions at the interface of stem cells and niche or descendent cells, paracrine and endocrine signals from local or distant sources, neural input and metabolic products of tissue activity.

interleukin-8 (IL-8). The kinetics of mobilization by these factors varies markedly, reflecting differing mechanisms; however, one mechanism not appreciated until recently is the ability of G-CSF to modify niche cells. G-CSF is capable of altering osteoblast activity sufficiently to reduce production of CXCL12, despite the absence of G-CSF receptors on these cells³⁸. It was an elegant, if serendipitous, discovery that mice with altered sympathetic nervous system function lack the ability to mobilize stem cells from the bone marrow in response to G-CSF³⁹. Sympathetic nervous system input, whether induced by G-CSF or by neural agonists, modulates the niche sufficiently to change the localization of stem cells. As the nervous system is inherently a circuit capable of connecting anatomically disparate tissues, these studies indicate one potential means by which the stem cell might be able to 'sense' changes in the state of the tissue or the organism. If there are changes, such as distant injury, neural connections might mediate the response even at the stem-cell level.

Besides the nervous system, an obvious ready means of connecting localized sites with information from a distance is the circulatory system (Fig. 3). HS cells might directly exploit their frequent blood trafficking to 'survey' input at a distance and some other stem-cell types might do the same^{40–42}. More likely is the physical association of stem cells with the microvasculature. In development, the co-localization of endothelial cells and progenitor populations from tissues as diverse as pancreas, heart, brain, liver, adrenal, bone and blood reflect the potential for regulatory signals generated by endothelium or nascent vasculature to affect primitive cell fate (reviewed in ref. 43). The adult hippocampus continues to generate neurons from precursor populations in perivascular loci that contain proliferating neural precursors, angioblasts and glial cells⁷. This region has been called an 'angiogenic niche'. Similarly, HS cells have been observed in abundance in close physical association with bone-marrow microvessels using recently defined immunohistochemical reagents⁸. The sites on such vessels where primitive cells engage and diapedese seem to be highly discontinuous, and are functionally distinguished by abundant CXCL12 and E-selectin expression⁴⁴. Although cells seem to reside there, only indirect data suggest that this represents a location that is regulating stem-cell function rather than just serving as a way-station for highly mobile cells. It is likely that the perivascular site is indeed a niche; however, at present, the data on function that are necessary to confirm this conclusion are lacking for stem cells. The haematopoietic cells known to be regulated in association with marrow vessels are committed progenitors⁴⁵.

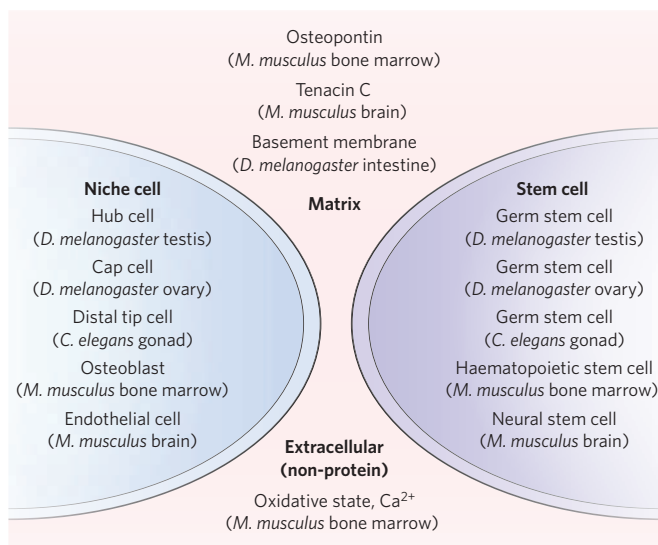


Figure 2 | Elements identified in stem-cell niches from various organisms and tissues. Intentionally excluded are the complex molecular interactions that are present at the interface between the stem cell and its niche cell counterpart. These are reviewed extensively elsewhere^{54,55}. *C. elegans*, *Caenorhabditis elegans*; *D. melanogaster*, *Drosophila melanogaster*; *M. musculus*, *Mus musculus*.

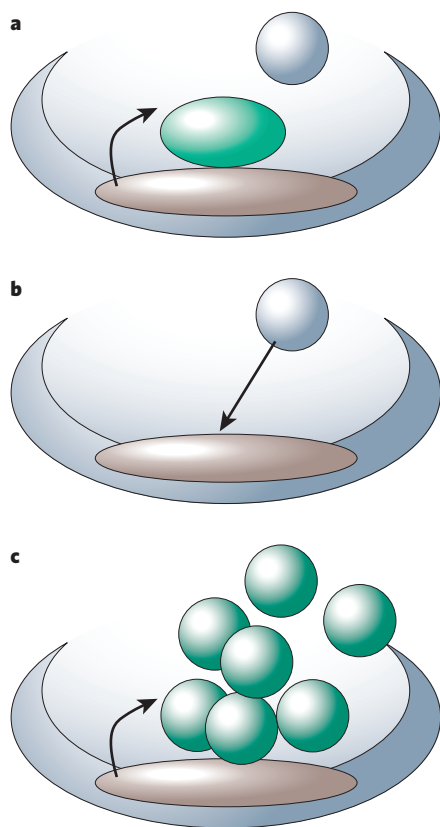


Figure 4 | Potential niche contribution to dysplastic cell growth. **a**, Normal interactions and occupancy of the niche tightly controls stem-cell number. **b**, Vacancy in the niche might result in ectopic occupancy by another cell type. **c**, Niche signals might impose proliferative or de-differentiative signals, contributing to a disordered state of tissue organization and control.

Although association with microvessels is an intuitively compelling means for a stem cell to be in an environment where system-wide signals can be sampled, the exact components of the vasculature that engage stem cells are still unclear. In certain circumstances, such as islet-cell proliferation, the basement membrane may again provide regulatory information⁴⁶. Endothelial cells have been implicated as directly inducing neural precursor Notch activation with resultant cell proliferation and differentiation in a manner that vascular smooth muscle cells do not⁴⁷. Pericytes surrounding the endothelium might contribute to stem-cell regulation, and have been found to be closely associated with mesenchymal stem cells⁴⁸. Endothelium, pericytes and surrounding smooth muscle are all candidate contributors to a perivascular niche for normal stem cells. They might also contribute to the foci of tumour-initiating or cancer stem cells thought to be responsible for blood-borne metastases. Recently, it was shown that such a site could be rendered more conducive to cancer-cell engraftment if modified by intravascular delivery of bone-marrow-derived vascular endothelial growth factor receptor 2 (VEGFR2)-positive cells⁴⁹. The idea that stem-cell niches can be modified or created has experimental support in other systems as well.

Under conditions of stress, such as fibrosis of the bone-marrow space, extramedullary haematopoiesis develops in humans and mice. Sites such as the spleen might re-kindle a developmental vestige (particularly in the mouse, in which the spleen is a haematopoietic organ) or engage entirely new sites, such as lymph nodes or soft tissue. These seem to function as regulatory niches for stem cells. Experimentally, niches can be generated by altered expression of single genes. Stabilized β -catenin expressed under the control of an epidermal-specific promoter resulted in *de novo* generation of hair follicles with a presumed attendant stem-cell pool⁵⁰. Therefore, niches are not static in function or number, and can be created or made to change under specific conditions.

What can a niche change?

The variability in niche number and function implicitly raises the issue of whether such modifications can participate in disease states or be induced to achieve therapeutic outcomes. Although altering normal stem-cell function is clearly one potential effect of altered niche dynamics, another more ominous possibility has been raised by lessons learned in *Drosophila*. Experimentally-induced stem-cell vacancy in the ovarian germ stem-cell niche did not result in immediate loss of niche structural integrity or potential. Rather, the empty niche could be occupied by somatic cells and, if not terminally differentiated, ectopic proliferation could ensue⁵¹. Furthermore, altered expression of a niche product, such as DPP, could induce more mature progenitor populations (cystocytes in *Drosophila*) to revert to a stem-cell phenotype⁵². A stem-cell state could be imposed upon more mature cells. Although neither of these scenarios has been explored in vertebrate systems, these mechanisms could be predicted to result in niche-generated disease. Indeed, altering cells to acquire more stem-like features is consistent with recent applications of stem-cell biology to the understanding of cancer. The stem-cell paradigm might be more than just a means of understanding how malignant tissue is organized and how cancer might be modified by a specific microenvironment. It raises the real possibility that a dysfunctional microenvironmental niche might contribute to the emergence of the disease (Fig. 4).

Modifying the niche

If the niche is a dynamic structure that has the capacity to be formed anew, to respond to exogenous signals and to include varying components, this raises the question of whether the niche can be a target for therapeutic manipulation. There are compounds defined for use in other settings that could be considered for 're-purposing' to affect the niche. Certainly, within the haematopoietic system, the association of the stem cell with bone raises the possibility that compounds developed for the modification of bone or mineral metabolism might have a role. Candidates include drugs that affect the osteoblast or osteoclast function of inhibiting molecular regulators of stem-cell interactions with the niche, such as drugs targeting the CaR and OPN receptors, or Notch. Experimental evidence has shown that altering the niche pharmacologically can change stem-cell outcomes, with physiologic impact. For example, the use of parathyroid hormone (PTH) to stimulate the receptor on osteoblasts resulted in an increase in the number of stem cells under homeostatic conditions. When the setting was the physiologic stress of bone-marrow transplantation, dramatic results were noted. The animals receiving PTH had markedly improved marrow cellularity and increased rates of survival⁵. The stem cells do not have PTH receptors, so the effects are indirect and probably mediated by niche osteoblasts. This effect has been shown to protect resident HS cells from cytotoxic damage during exposure to chemotherapy drugs, to improve harvests of stem cells from treated animals and to improve engraftment outcome. Whether this paradigm of identifying characteristics of a niche to permit rational pharmacologic manipulation can be applied to other stem-cell systems needs to be tested further. However, if demonstrated, this would offer a novel approach to regenerative medicine.

The niche as a target in cancer therapy

With the recent development of the cancer stem-cell concept, which was first proposed half a century ago but was only empirically shown by the pioneering work of Dick and others, another dimension of the niche as a 'druggable' target is beginning to take shape⁵³. If cancer is organized in a manner similar to normal tissue, with a minor subpopulation of stem cells, an attendant blood supply and a unique microenvironment, there might be a similar dependence of the stem cells on a cancer niche. The possibility of a niche has been shown by multiple studies indicating distinctive features of non-cancerous cells within a tumour, and by the recent report of a population of bone-marrow-derived cells paving the way for subsequent establishment of

a tumour focus. If the components of this niche could be demonstrated and targeted, it would be of considerable interest to modify the relative support of the stem-like cells of cancer. The idea that the niche might be a druggable target would be extremely appealing as an adjunctive and entirely independent means of targeting malignant cells. Perhaps the niche will be more than a biologist's puzzle, and will become a guide for novel therapies to enhance the regenerative capacity of normal stem cells and limit the malignant potential of cancerous ones. ■

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Stem cells, ageing and the quest for immortality

Thomas A. Rando¹

Adult stem cells reside in most mammalian tissues, but the extent to which they contribute to normal homeostasis and repair varies widely. There is an overall decline in tissue regenerative potential with age, and the question arises as to whether this is due to the intrinsic ageing of stem cells or, rather, to the impairment of stem-cell function in the aged tissue environment. Unravelling these distinct contributions to the aged phenotype will be critical to the success of any therapeutic application of stem cells in the emerging field of regenerative medicine with respect to tissue injury, degenerative diseases or normal functional declines that accompany ageing.

*The woods decay, the woods decay and fall,
The vapours weep their burthen to the ground,
Man comes and tills the field and lies beneath,
And after many a summer dies the swan.
Me only cruel immortality
Consumes;*

from *Tithonus*, by Alfred Tennyson

Stem cells have captured the popular imagination with the promise of enhanced tissue repair, the treatment of degenerative diseases and even the amelioration of dysfunction associated with normal ageing. The possibility that stem cells could have therapeutic applications in the context of normal ageing raises two related questions. First, what is the effect of ageing on stem cells themselves? Second, to what extent can the functional decline of a tissue be attributed to a limited (and perhaps declining) capacity of the resident stem cells to maintain normal tissue structure and function?

The first question has been amenable to study in specific cases. As a starting point, one needs to identify and isolate stem cells, assess their numbers and functionality, and then apply those assays across the lifespan to test for changes with age. The real challenge is then to try to understand the causes and mechanisms of those changes. The second question is much more difficult to address experimentally because of the tight link between stem cells and the tissues in which they reside. As such, there are no tissues for which this question has been definitely answered. Furthermore, the issue is vastly different depending on whether the tissue exhibits high cellular turnover, low cellular turnover but high regenerative potential in response to damage, or low cellular turnover and negligible regenerative potential (Fig. 1). The role of stem cells in the biology of these different classes of tissues is so distinct that the extent to which ageing of the stem-cell compartment might affect the tissue cannot be extrapolated from one tissue to another. The focus of this review is the intersection between the biology of ageing and the biology of adult stem cells, paying particular attention to mammalian tissue biology, functional changes with age in those tissues that might or might not relate to stem cells, and the relationship between tissue ageing and longevity.

Ageing, evolution and stem-cell immortality

Evolutionary theories of ageing

The study of how stem cells change with age leads inevitably to the underlying question, which can only be couched in evolutionary terms, of why we age. The value of asking this question is not to unravel a certain purpose of ageing, but rather to frame the processes of ageing within evolutionary constraints that might have been imposed by

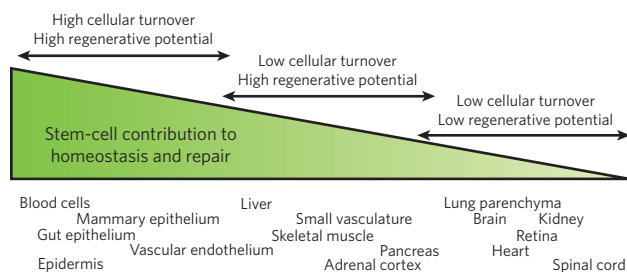


Figure 1 | Tissue heterogeneity and stem-cell functionality for homeostasis and repair. The extent to which the effects of ageing on the resident stem cells determine the phenotype of an aged tissue is likely to correlate with the extent to which stem cells are responsible for normal tissue homeostasis and repair. Along this spectrum, tissues generally fall into one of three categories. First, tissues with high turnover (such as blood, skin and gut) have a prominent stem-cell compartment and, by definition, have high regenerative capacity. Second, tissues with low turnover but high regenerative potential might use different strategies to ensure effective repair in the setting of acute injury. In skeletal muscle, for example, differentiated myofibres are unable to proliferate to generate new tissue, so muscle must rely on resident stem cells for all turnover and repair⁶³. For the liver, it seems that differentiated hepatocytes can proliferate sufficiently to mediate effective tissue remodelling, repair and replacement normally⁶⁴, whereas stem cells might be recruited in the setting of severe injury⁶⁵. Third, tissues with low turnover and low regenerative potential might have stem cells that mediate only limited tissue repair. Although there is much interest in harnessing the potential of stem cells in the brain⁶⁶ and heart⁶⁷ for therapeutic purposes, for example, there is limited endogenous repair capacity of these tissues following acute injuries.

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selective pressures. Evolutionary theorists have entertained the possibility that ageing is genetically programmed, based on the notion that elimination of individuals who are past their reproductive prime would be beneficial for the species, as this would theoretically preserve resources for the most reproductively fit individuals and for future generations. However, this view has not withstood rigorous analysis, primarily because the 'aged phenotype' is rare in nature¹. The majority of animals in the wild succumb to starvation, predation, exposure, disease or accident long before the appearance of characteristics that are recognized as ageing in humans and in domesticated and laboratory animals.

Two theories have emerged that integrate the possibility of genetic programmes that direct the ageing process with the notion that such programmes are not likely to have arisen by pressures of natural selection². The first is the 'mutation accumulation' theory, first proposed by Medawar³, which posits that mutations leading to detrimental age-related changes could accumulate over successive generations if their effects were only realized well after the age of peak reproductive success. As few individuals would actually reach those ages, such mutations would escape negative selective pressure. The second theory, the 'antagonistic pleiotropy' theory⁴, postulates that there might be genes whose expression is harmful in later life that accumulate in populations not because they are silent earlier in life but because they are actually beneficial to survival or reproductive fitness. Such mutations could thus have a selective advantage. Although these concepts have guided attempts to merge evolutionary theory with empirical studies of the biology of ageing, little evidence of cumulative mutations, and only rare examples of genes that display antagonistic pleiotropy, have been found^{2,5}.

Nevertheless, there is overwhelming evidence that there are strong genetic influences on the rate of ageing. Perhaps the most compelling evidence is that the differences of rates of ageing within individuals of a species are negligible compared with the vast differences across species⁶. A mayfly (a member of the aptly named order Ephemeroptera) moults, reproduces and dies within a single day, in some cases with a functional lifespan measured in hours; by contrast, giant tortoises can live for almost 200 years (Fig. 2). The powerful influence of genetics is further reflected by the ever increasing number of single-gene mutations that can influence the lifespan of eukaryotes ranging from yeast to mice^{7–13}.

A theory that provides a more generalizable framework for considering the influences of genetics on the ageing process is the 'disposable soma' theory¹⁴. A key aspect of this theory is the distinction between the immortal germ line and the 'disposable' (that is, mortal) soma. The basic premise is that species have evolved with genetic programmes that optimize the utilization of resources for survival and reproduction. Ageing occurs through 'wear and tear', and the genetic programme of any species is designed to resist that damage long enough for organisms to reach reproductive maturity. Any increased environmental stress that

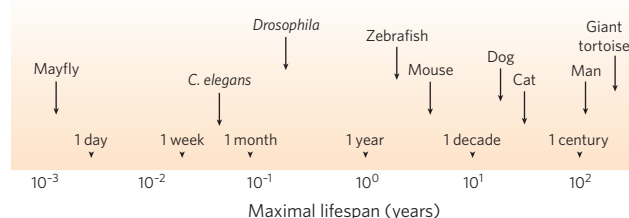


Figure 2 | Variation of maximal lifespan across species. Presented on a logarithmic timescale, this figure illustrates the vast range of maximal lifespans at the extremes (mayfly and giant tortoise), in some of man's favourite pets (dog and cat) and experimental animals (*Caenorhabditis elegans*, *Drosophila*, zebrafish and mouse), and in man himself.

Box 1 | Self-renewal: a misnomer and an impossible standard to meet

One characteristic that is demanded of a cell to be admitted into the stem-cell pantheon is the ability to self-renew. Is this fair? It depends on how rigorously applied the definition of self-renewal is. Clearly, no cell could meet the standard if self-renewal were defined as cell division without any errors of DNA replication. Yet, if such subtle molecular differences between mother and daughter cells are allowable within the definition of self-renewal, what other differences are also allowable? Clearly, for practical purposes, the issue of self-renewal is a physiological one. Does the daughter cell possess two key functional features of the mother cell, namely, the ability to generate the same spectrum of differentiated progeny and the ability to generate another daughter cell that can also generate the same spectrum of differentiated progeny? However, just as too rigorous a molecular definition renders self-renewal impossible to achieve, even this looser physiological definition requires that the criteria are not applied too stringently. For example, is it a failure of self-renewal if the daughter cell can generate the same types of differentiated progeny as the parent but fewer of them, or if the daughter cell's progeny, when induced to differentiate, are skewed towards one particular differentiated phenotype?

Within the context of ageing, this gradual change of genotype and phenotype across generations, or 'replicative ageing', is superimposed upon the chronological ageing that occurs in any cell with the passage of time (Fig. 5) — a distinction that has been elegantly made in ageing studies of yeast⁶². In either replicative or chronological ageing, genetic drift through mutation accumulation governs intrinsic stem-cell ageing and affects stem-cell functionality. These kinds of changes directly pertain to two levels of analysis that are commonly used to characterize stem cells: the pattern of expression of proteins by flow cytometry or immunocytochemistry, and the pattern of transcript expression by microarray analysis. The important issue is not whether the patterns detected in the progeny differ from the patterns detected in the parental population, but rather whether there is any functional loss. The renewal of function supersedes any definition of self-renewal at a molecular level. This is not to discount the value of markers for identifying stem cells and more committed progeny; rather, it is a caution against having too stringent criteria for self-renewal, particularly with regard to molecular determinants.

challenged the soma would divert limited resources away from reproduction. This is consistent with the well recognized, although not obligatory, negative correlation between lifespan and fecundity¹⁵. This theory also provides a framework for integrating the effects of caloric restriction on prolonging lifespan and reducing fecundity¹⁵, and the intriguing fact that so many of the single-gene mutations that prolong lifespan are involved in pathways of energy metabolism and stress responses^{8,9,12,13,16}.

The disposable soma theory also provides an interesting, if unanticipated, framework for considering adult stem cells in relation to both the disposable soma in which they reside and the immortal germ line. Are adult stem cells more similar to the rest of the soma or to the cells of the germ line?

The immortal germ line and the mortal soma

Although the distinction between the mortal soma and the immortal germ line is unambiguous in mammalian species, it is less clear across a broader phylogenetic spectrum. Seemingly unlimited regenerative capacity of appendages and limbs is seen in organisms such as salamanders and starfish^{17,18}. This regeneration of body parts does not produce new individuals in these species, but it does begin to hint at the possibility. It is in organisms such as planaria and hydra that 'somatic' parts are capable of giving rise to entirely new individuals^{19,20}, which is more reminiscent of the immortality of the germ line or of single-celled organisms that divide by fission.

The concept of immortality differs when considering the germ line, the early cells of the embryo that can be derived as embryonic stem (ES) cells and studied *in vitro*, and the soma. For the germ line, immortality is dependent on adaptive change and natural selection promoting survival and reproductive success associated with advantageous

changes in the genome, and is gauged over evolutionary time. For ES cells, the criteria for immortality are determined by the investigator. Progeny with changes that would confer a selective growth advantage are deemed unfavourable and discarded because they are usually the result of karyotypic changes²¹. Thus, immortality of stem-cell lines is assured by selecting progeny with properties as close to the parental cells as possible, not by the natural selection of competitive reproductive advantage. Still, genetic drift will inevitably occur over time and immortality of the line might be measured in decades, not eons. For the soma, the conventional meaning of immortality is ‘living forever’, which speaks more of mythology than of science. Nevertheless, the prospect of increasingly long-lived individuals emerges from scientific study of lifespan and its extension. One is, however, reminded of the Greek myth of Tithonus, a mortal who became the lover of the goddess Eos. When Zeus stole away another of Eos’s mortal lovers, he promised to fulfil one wish to repay her. Eos asked Zeus to grant Tithonus immortality. Unfortunately, she forgot to include eternal youthfulness in her wish. Oops. As a result, although Tithonus was given the ‘gift’ of immortality, he continued to age, withered beyond recognition and ended up begging for death for all of eternity.

A feature of germline immortality that is important for adult stem cells is the ability to ensure that genetic information is passed on with the highest fidelity to successive generations. Cells must have robust mechanisms to resist and/or repair damage to the genome²². For ES cells, the maintenance of genome stability is essential to their value as tools for research and potential therapeutic vehicles²³. For adult stem cells *in vivo*, possessing the capacity to resist, detect and repair changes in the genome (such as telomere shortening and mutation accumulation) underlies their ability to participate in tissue homeostasis and repair across an organism’s lifespan. Fundamentally, this is at the heart of one of the key features that defines stem cells, namely, the ability to self-renew, although the extent to which maintaining genomic integrity is imperfect means that the criteria for self-renewal must not be too stringent (Box 1). Finally, the possibility that many forms of cancer might be the result of acquired mutations in adult stem cells highlights an even more important consequence, at least in terms of health, of a failure to maintain genomic stability in adult stem cells²⁴.

The ageing process and adult stem-cell functionality

The hierarchical nature of biological defences

The process of organismal ageing is characterized by functional decline due to histologic and biochemical changes in tissues and organ systems with the passage of time. Declining functionality is paralleled by diminishing capacity to respond to injury or stress. As stem cells are involved in homeostasis as well as regeneration and repair for many tissues, the question naturally arises as to whether the characteristics

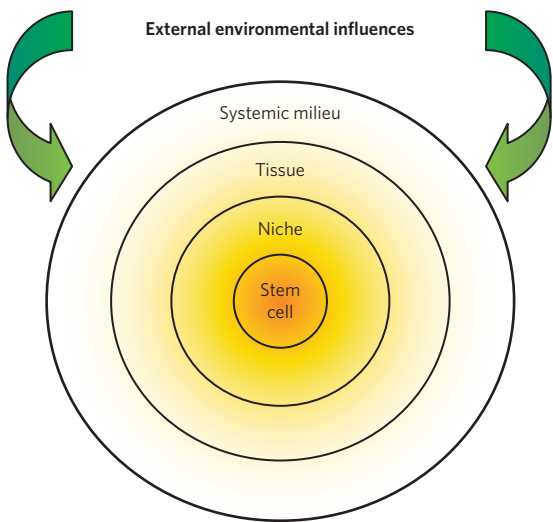


Figure 3 | Influences on stem-cell functionality. Adult stem cells interact dynamically with their environment at increasing levels of complexity. Locally within their niche, stem cells respond to the extracellular matrix, to other cells within the niche and to paracrine factors. Within the tissue, the stem cell/niche unit is influenced by soluble factors derived from parenchymal cells, which are, in turn, influenced by systemic immunological and neuroendocrine signals. Finally, external environmental influences, which are particularly relevant to discussions of ageing, filter down through these various levels to influence stem-cell function.

of an ageing tissue can be understood as a declining functionality of the adult stem cells that reside within it. However, this question would seem to place stem cells in a unique position as guardians of tissue youthfulness, rather than within the hierarchy of homeostatic mechanisms that decline with age, ranging from the molecular to the organismal (Table 1). The accumulation of mutations in nuclear and mitochondrial DNA, despite the range of repair mechanisms for preventing such accumulation²⁵, sits at the pinnacle of the hierarchy, representing the most fundamental and irreversible changes from which many others follow. Stem cells contribute at an intermediate level of tissue homeostasis and repair, and, as noted above (Fig. 1), might contribute negligibly to the ageing phenotype for tissues with extremely low cellular turnover. In the heart and brain, for example, where the overwhelming majority of cardiomyocytes and neurons, respectively, are not replaced during adult life, the aged phenotype is best understood in terms of changes in these postmitotic cells rather than any stem-cell compartment.

Table 1 | Manifestations of ageing and homeostatic defences

Position in hierarchy	Manifestations of ageing	Homeostatic mechanisms/defence
Molecular changes that lead to cellular dysfunction	Cumulative mutations in nuclear and mitochondrial DNA	DNA repair activities; telomerase activity
	Oxidative damage to cellular constituents	Antioxidant enzymes, cytosolic and membrane free-radical scavengers
	Accumulation and aggregation of abnormal proteins, lipids and other macromolecular constituents	Mechanisms to recognize and degrade abnormal proteins and other macromolecules
Cellular changes that lead to tissue dysfunction	Cell death	Anti-apoptotic pathways
	Oncogenesis	Cell-cycle checkpoints, tumour-suppressor genes, apoptosis pathways
	Senescence	Immune surveillance
Tissue changes that lead to organismal dysfunction	Atrophy from cell loss and diminished regenerative capacity	Stem cells for tissue maintenance and repair
	Extracellular matrix changes	Matrix remodelling activities such as those of metalloproteinases
	Extracellular deposits	Phagocytic activities of resident and circulating cells

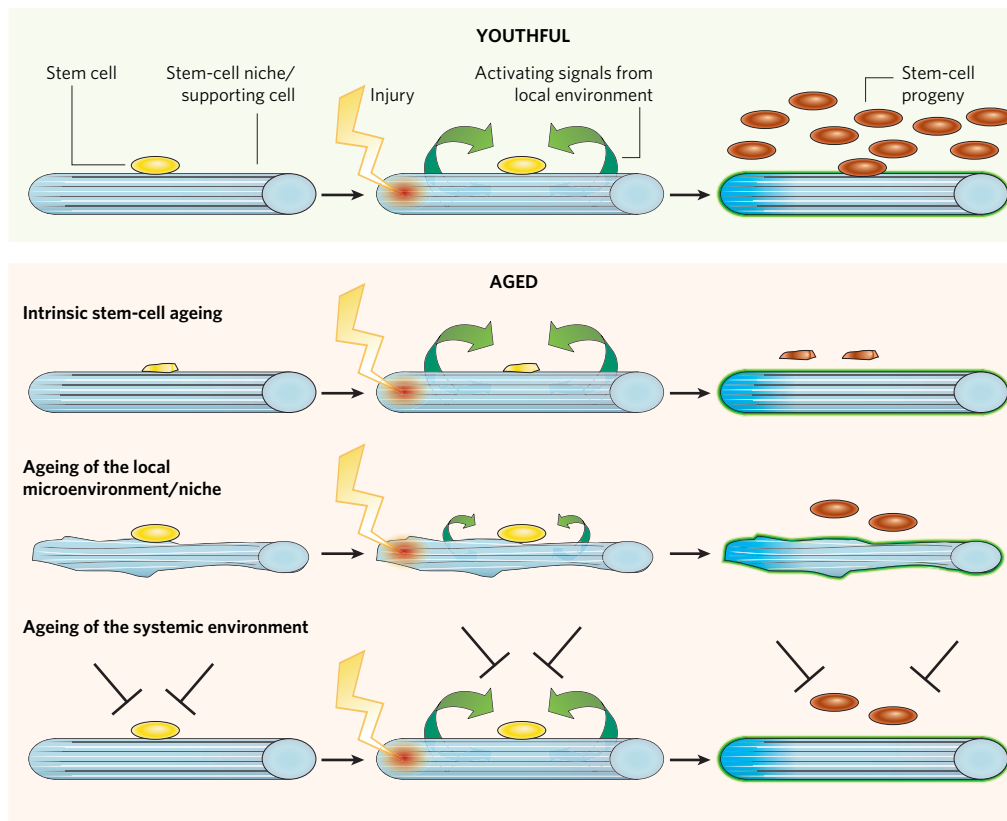


Figure 4 | Ageing of stem-cell functionality. The decline in stem-cell functionality with age can be due to age-related changes at many levels. This figure illustrates several possibilities using a skeletal muscle fibre and an associated stem (satellite) cell as a model. In response to tissue injury, local signals induce satellite cells to begin proliferating in order to generate sufficient progeny for tissue repair. Age-related changes in satellite cells, in the satellite-cell niche or in the systemic milieu could all result in a diminished functionality of satellite cells in an aged organism, manifested as a decreased propensity to generate sufficient functional progeny for effective regeneration.

Stem-cell functionality and the stem-cell niche

Almost every tissue studied has shown age-related decrements in the rate and/or efficacy of normal cellular turnover and regeneration in response to injury. Whereas this strongly suggests an age-related decline in stem-cell functionality, it does not necessarily imply that there is intrinsic stem-cell ageing. Stem-cell function is regulated at increasing levels of complexity, from cell-autonomous regulation to regulation by the local environment of the stem-cell niche, the surrounding tissue, the systemic milieu of the organism and, ultimately, the external environment (Fig. 3). Furthermore, there are interactions among these compartments such that the systemic milieu is the product of all the various tissue/niche/stem-cell units throughout the organism. Declining tissue homeostasis or repair could arise from age-related changes in the numbers or properties of stem cells, in the local environment or niche in which the stem cells reside, in the systemic milieu of the organism that influences all cells, or in any combination of these. Changes within the niche would include alterations in the amount and composition of the extracellular matrix, changes in the membrane proteins and lipids in cells that make direct contact with stem cells, and changes in soluble paracrine and endocrine factors that constitute the systemic milieu. Studies of the importance of such changes in the stem-cell niche have been best described in the germline stem-cell niche in *Drosophila*^{26,27}. Systemic changes would include immunological and neuroendocrine changes, and, in the case of tissue injury or disease, changes in the factors that are released from damaged cells and in the inflammatory response that accompanies such damage. These effects have been demonstrated in heterochronic transplantation or parabiosis studies in which cells from aged animals are exposed to systemic factors of young animals (and *vice versa*)^{28–31}. Thus, even in the absence of significant ageing of stem cells themselves, stem-cell functionality could show a marked age-related decline due to decrements in the signals within the local and systemic environment that modulate the function of stem cells or their progeny (Fig. 4).

Stem cells and longevity

Even more enigmatic than the role of stem cells in tissue ageing is the relationship between stem cells and longevity itself. There is no evidence

that the lifespan of any species is determined by a limited supply or limited functionality of its stem-cell populations, and yet this association is commonly made^{32,33}. There is a risk of tautologies if one starts with the premises that declining stem-cell function is responsible for tissue ageing and that tissue ageing determines longevity. A major weakness of the first premise is the negligible role of stem cells in the ageing of tissues with low cellular turnover. The second premise fails to acknowledge the experimental and conceptual gap that exists between our understanding of tissue ageing and the determinants of lifespan. Common sense dictates that there must be a relationship between the two, and experimental interventions that alter the lifespan of model organisms also tend to alter tissue ageing, but the direct link remains elusive. It is clear that single-gene mutations that extend maximal lifespan also result in structural and physiological changes in tissues that indicate a slowing of the ageing process and a reduction in age-related pathologies^{12,13}. Likewise, the increased longevity from caloric restriction in various species is accompanied by an apparent reduction in the rate of tissue ageing^{34–37}. However, none of these important advances in the biology of ageing provide any direct evidence as to why aged individuals within a species die in the absence of identifiable injury or disease. The cause of death from 'old age' remains one of the central mysteries of biology (Box 2). However, there is no evidence that the maximal lifespan of any species is determined by declining stem-cell function or, conversely, that increasing the number or functionality of any single stem-cell population would extend lifespan. One need only look at species with tissues that are almost entirely postmitotic to see that there cannot be any tight mechanistic link between adult stem-cell function and longevity in any evolutionary sense. Longevity *per se* is several steps removed from the biology of stem-cell ageing, linked primarily by how genetic variations and environmental factors might influence each, perhaps coordinately, but not by any demonstrated causal relationship.

Tests of intrinsic ageing of adult stem cells

Given the inherent complexity of distinguishing intrinsic cellular ageing from ageing of the cellular milieu when stem cells are studied in their native environment, the most direct tests of intrinsic cellular

ageing have been those that have involved isolation of stem cells from young and old animals, and transfer to identical *in vitro* or *in vivo* environments. This has been done extensively for only a few stem-cell populations. An important distinction even using these more rigorous assays of stem-cell ageing is whether any cell-autonomous changes that are detected are stable and heritable, or if they are reversible. In some cases, stem cells from aged animals show a delay in responsiveness to activating stimuli, ultimately yielding comparable results to those obtained from young stem cells, suggesting that the initial responses might reflect epigenetic modifications rather than irreversible (at least under physiological conditions) genetic or biochemical changes. Generally, the effects of age on isolated stem cells are compared in assays of growth, differentiation, apoptosis, transformation and senescence. Although cellular senescence has been characterized primarily as an *in vitro* phenomenon related to replicative lifespan^{38,39}, it has also been proposed that cellular senescence occurs *in vivo* and might result in a toxic 'gain of function' by promoting an environment that fosters cellular transformation⁴⁰.

Two specific examples of adult stem cells — haematopoietic stem (HS) cells and skeletal muscle satellite cells — are highlighted below because they have been prospectively isolated, and the effects of ageing have been studied both *in vivo* using transplantation assays and *in vitro*. In addition, although both populations are derived from tissues with high regenerative potential, they also represent opposite ends of the spectrum of tissues in terms of normal cellular turnover (Fig. 1). As such, the stem cells and their progeny in blood will be much more affected by the combined effects of replicative and chronological ageing than will the stem cells and their progeny in skeletal muscle, which are likely to experience primarily chronological ageing (Fig. 5).

Haematopoietic stem cells

These give rise to the cellular constituents of blood that serve life-sustaining functions, such as oxygen transport, blood coagulation and immune function. With age, there is evidence of a gradual decline in all of these functions^{41,42}. Such changes are not due to the gradual depletion of HS cells, as there is actually an increase, rather than a decrease, in HS cells with age⁴³. *In vitro* studies of isolated HS cells have shown that there

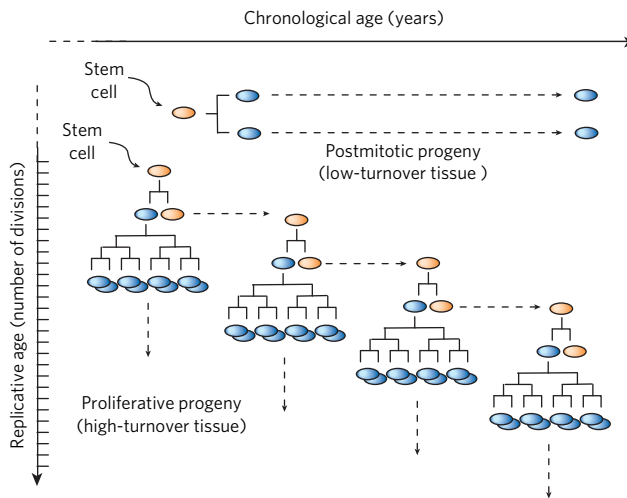


Figure 5 | Chronological and replicative ageing. Attempts to determine the age of a cell are confounded by the distinction between chronological and replicative ageing. Certain stem cells may give rise to postmitotic cells that are present in the tissue for the entire lifespan of the organism. Such postmitotic cells experience primarily chronological ageing. To the extent that the stem cells within those tissues remain quiescent, they would also experience mainly the effects of chronological ageing. By contrast, certain stem cells, particularly those in tissues with high turnover, divide regularly and generate progeny that undergo extensive proliferative expansion. There is an additional component of ageing for those stem cells and their progeny — that is, replicative ageing. On the basis of the evidence that, depending on the species, mammalian somatic cells have a limit to their replicative potential related to genomic instability from telomere shortening, mutation accumulation and genomic rearrangements⁶⁸, proliferative cells will integrate the effects of chronological ageing with the effects of replicative ageing.

is no difference between young and old cells in terms of their ability to form colonies and their proliferative potential⁴⁴. Likewise, there does not seem to be any decline with age of the ability of HS cells to interact with stroma *in vitro*⁴⁴.

When HS cells have been tested in serial-transplantation experiments, complete reconstitution of the blood occurs over several lifespans in mice⁴⁵, and old HS cells are as effective as young HS cells at reconstituting the blood lineages after transplantation^{28,46,47}. In addition, the size of individual stem-cell clones in recipients receiving single competitive-repopulation units is independent of age⁴⁸. However, aged HS cells seem to be less effective at homing and engrafting⁴⁴, suggesting that intrinsic ageing of HS cells can be revealed by this type of analysis. The extent of intrinsic ageing of HS cells also seems to be strain dependent, as determined by competitive-repopulation studies^{46,49}. These cell-intrinsic changes might be epigenetic, as they could be reversed when the cells were placed in the appropriate environment⁵⁰. In these assays, cells of different genetic backgrounds responded differently, highlighting the importance of genetic influences on the ageing process. Recently, direct molecular analysis of purified HS cells revealed differences in gene expression between cells from young and old mice⁵¹. It is intriguing that the changes in specific genes were correlated with the noted skewing of the lineage potential of aged HS cells⁵². It will be interesting to test whether these cell-intrinsic changes are also reversible and dependent on the environment in which the cells reside.

Skeletal muscle satellite cells

The primary skeletal muscle stem cell (the satellite cell) is quiescent in adult muscle, but is activated to proliferate and generate committed progeny in response to injury or disease⁵³. Estimates of satellite cell number with age vary depending upon the method used, ranging from a slight increase⁵⁴ as reported for HS cells, to a minimal change⁵⁵ or a decline^{54,56}. However, there is a much greater impairment of regenerative potential with age than can be accounted for by any decline in number⁵⁷.

Box 2 | Dying of old age

There is no compelling explanation for the cause of death in old but otherwise healthy humans, mice, worms or flies, or any other organism for that matter. The colloquial expression 'dying of old age' belies our knowledge of the biological basis of this event. Surely, the cessation of respiratory and circulatory functions results quickly in irreversible damage to vital organs; however, to insist that ageing of the heart or lungs is the cause of death only sidesteps the question. Examination of tissues of an old member of a species at the time of death will reveal stereotypical biological changes and perhaps even pathological changes that were only mildly symptomatic or even asymptomatic. Why, then, did this individual die? We can measure average and maximal lifespan in species, we can evaluate the effects of genetic, nutritional or pharmacological interventions that alter those indices, and we can correlate them with changes in tissue ageing. Yet no hypothesis has emerged that yields a useful definition of dying of old age in terms of cell and tissue biology. In the absence of an acute event or an overwhelming disease, the ageing process affects all tissues and cells, whether they are postmitotic, actively mitotic or quiescent. The result is a recognizably progressive change for which the actuarial definition of increased probability of dying corresponds to empirical biological and physiological changes, but fails to provide any clue as to the mechanism. The question 'When does life begin?' engenders much debate, and the biological mechanisms that underlie the processes suggested as answers — for example, fertilization, gastrulation and implantation — are intensely studied. Although much effort has gone into defining death from a medical and legal perspective, little effort has been devoted to studying the processes that lead to 'death from old age', and rarely is the question asked from a biological perspective, 'When does death begin?'

This age-related decline of regenerative potential has been attributed to impairment in aged muscle of the Notch signalling pathway, which is essential for normal satellite-cell activation in young animals⁵⁵.

Initial *in vitro* studies of satellite cells from young and old animals suggested that there was intrinsic ageing of this stem-cell population, as aged cells generated far fewer progeny^{58,59}. However, this interpretation was at odds with the finding that regeneration mediated by aged satellite cells was highly effective when the cells were transplanted into young animals as whole-muscle grafts³⁰. In fact, the results were indistinguishable from the regeneration mediated by grafting of young muscle. These data suggest reversible epigenetic modifications of aged muscle stem cells, an interpretation supported by recent data showing that aged muscle stem cells, when exposed to a youthful systemic milieu by virtue of parabiotic pairings of aged and young mice, activate and repair muscle nearly as well as young satellite cells³¹. Thus, it seems that the diminished regenerative potential of aged muscle is not primarily due to intrinsic ageing of satellite cells, but rather to the effects of the aged environment on satellite-cell function. Gene-expression studies of cells derived from satellite cells have shown changes with age⁶⁰. However, as with HS cells, it is not clear whether these transcriptional profiles are due to irreversible genetic changes or reversible epigenetic effects.

Fantasies and realities concerning stem-cell therapeutics

The area of health and ageing that is likely to benefit soonest from advances in the biology of adult stem cells is the emerging field of regenerative medicine. This could involve either the enhancement of endogenous stem cells or the transplantation of exogenous stem cells that have been expanded in culture. Transplanting exogenous stem cells into damaged tissues will be firmly founded on the principles of transplantation biology and immunology. However, an appreciation of the importance of the niche, especially the aged niche, will be critical to the success of stem-cell transplantation in enhancing tissue repair in the setting of acute injury. The importance of the host environment becomes even more complex when considering the use of stem cells in the treatment of chronic degenerative diseases.

Despite the historical quests for immortality and continuing interest in extending human longevity⁶¹, the application of stem-cell 'therapeutics' to delay the ageing process itself is even more remote. Given the universality of the ageing process and the profound influence of the systemic milieu on cells within a tissue, the theoretical basis of such an application is unclear. Any translation from animals to humans of experimental methods for increasing longevity is more likely to emerge from an understanding of the systemic coordination and regulation of cellular metabolic activity and cellular defences, although any extension of lifespan is sure to come at some cost.

Nevertheless, the potential for stem cells to be used as therapeutic vehicles has had a profound effect on the vision of the future of regenerative medicine. The suppression of adult stem-cell proliferation by the systemic milieu in aged animals, although it limits tissue regenerative potential and possibly promotes senescence or apoptosis, might be a crucial defence against the development of cancer, the likelihood of which increases with the accumulation of mutations in the stem-cell genome with age. Therapeutic applications of adult stem cells to aged tissue repair in the context of regenerative medicine will require an increased understanding of stem-cell biology, the environment of the aged tissue and the interaction between the two.

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Generation of neuronal variability and complexity

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The production of specialized differentiated neurons derived from stem cells has been proposed as a revolutionary technology for regenerative medicine. However, few examples of specific neuronal cell differentiation have been described so far. Although stem-cell tissue replacement might be seemingly straightforward in other cases, the high degree of complexity of the nervous system raises the challenge of tissue replacement substantially. Understanding mechanisms of neuronal diversification will not only be relevant for therapeutic purposes but might also shed light on the differences in cognitive abilities, personality traits and psychiatric conditions observed in humans.

The complexity of the human brain permits the development of sophisticated behavioural repertoires, such as language, tool use, self-awareness, symbolic thought, cultural learning and consciousness¹. Partly owing to the uniqueness of the neuronal heterogeneity and interconnections in our brains, each human is different. Indeed, the ability to perform high-level cognitive functions has been shown to be directly influenced by individual memory capacity, brain complexity and neural activity². Such complexity is the result of millions of years of evolution that have equipped neural progenitor cells in the embryo with the ability to generate every neuron in the brain — an extraordinary example of cellular potency. Other mammals, such as mice and chimpanzees, also have high levels of cellular heterogeneity, but the level of complexity in their brains is believed to be lower than that observed in humans. The mouse brain contains approximately 75 million neurons³. By contrast, the human brain is composed of approximately 100 billion neurons. The total number of synapses in the human neocortex is estimated to be 0.15 quadrillion (10¹⁵), and a typical neuron has approximately 5,000–200,000 synapses⁴. The diversity of single neurons provides the underpinning for how neuronal circuits operate; therefore, it is essential to identify the participating neuronal subpopulations, and to determine the functions of the neurons at the cellular and molecular levels.

An extraordinary diversity is found within the neuronal population. There might be as many as 10,000 types of neuron, although the definition of neuronal type is debated. Diversity exists between individual cells within a neuronal subtype, highlighting the importance of single neurons in the network. Even neurons with a similar morphology can differ in important molecular details, expressing different combinations of ion channels, for instance, providing cells with various excitation thresholds and distinctive firing patterns. However, how and when neuronal diversity is generated remains unknown. Here we review several molecular mechanisms that contribute to neuronal diversity.

Origins of neuronal diversity

Neurons of the mammalian central nervous system (CNS) arise during embryonic development from neuroproliferative zones surrounding the ventricles of the neural tube⁵. For a defined period of time, all types

of postmitotic neuron must be formed from precursor neuroblasts that exit the cell cycle and migrate from these regions to form the adult grey matter. During embryonic neurogenesis there is a massive proliferation of cells, but only 15–40% of post-migratory cells survive^{6,7}, suggesting that a selection process is occurring⁸. Interestingly, neurogenesis continues in the adult brain in discrete areas, such as the anterior part of the subventricular zone and the subgranular zone of the dentate gyrus (DG) of the hippocampus^{9–11}.

DNA rearrangement was first suggested to be responsible for the generation of neuronal diversity in a fashion similar to that seen in the somatic DNA rearrangement of the immune system, which is known as the V(D)J mechanism¹². In the immune system, the highly diverse array of antigen receptors can be attributed to the stochastic nature of the recombination process in somatic precursor cells, causing permanent changes in DNA and gene expression. This diverse population is then the target of selective processes that favour the correct antigen–receptor match and eliminate those with inadequate specificities, accounting for the rapid kinetics and immense diversity observed *in vivo*. In the nervous system, diversity is high during initial development¹³ and increases owing to activity in a more mature brain (Fig. 1). The activity-dependent neuronal diversity in postmitotic neurons is driven by environmental stimuli that dynamically refine neuronal networks, such as in the hippocampal ‘place cells’¹⁴. Yet how the original diversity between neurons is generated remains to be uncovered. Evidence for a possible similarity between the nervous and immune systems came from studies on mice deficient in DNA double-strand-break repair, revealing impaired neurogenesis as well as lymphogenesis, suggesting that normal neuronal development requires essential components of the conserved non-homologous end-joining pathway¹⁵. However, attempts to demonstrate a programmed recombination event in the CNS have led to unreconciled controversy^{16,17}. Rigorous but not definitive arguments against this hypothesis have come from experiments that used the genome of a single olfactory receptor neuron to clone an entire mouse (see page 1061); no evidence for recombination was found^{18,19}.

In the next sections, we review some of the candidate molecular mechanisms that can actively contribute to the generation of neuronal diversity, acting on DNA, RNA and protein substrates.

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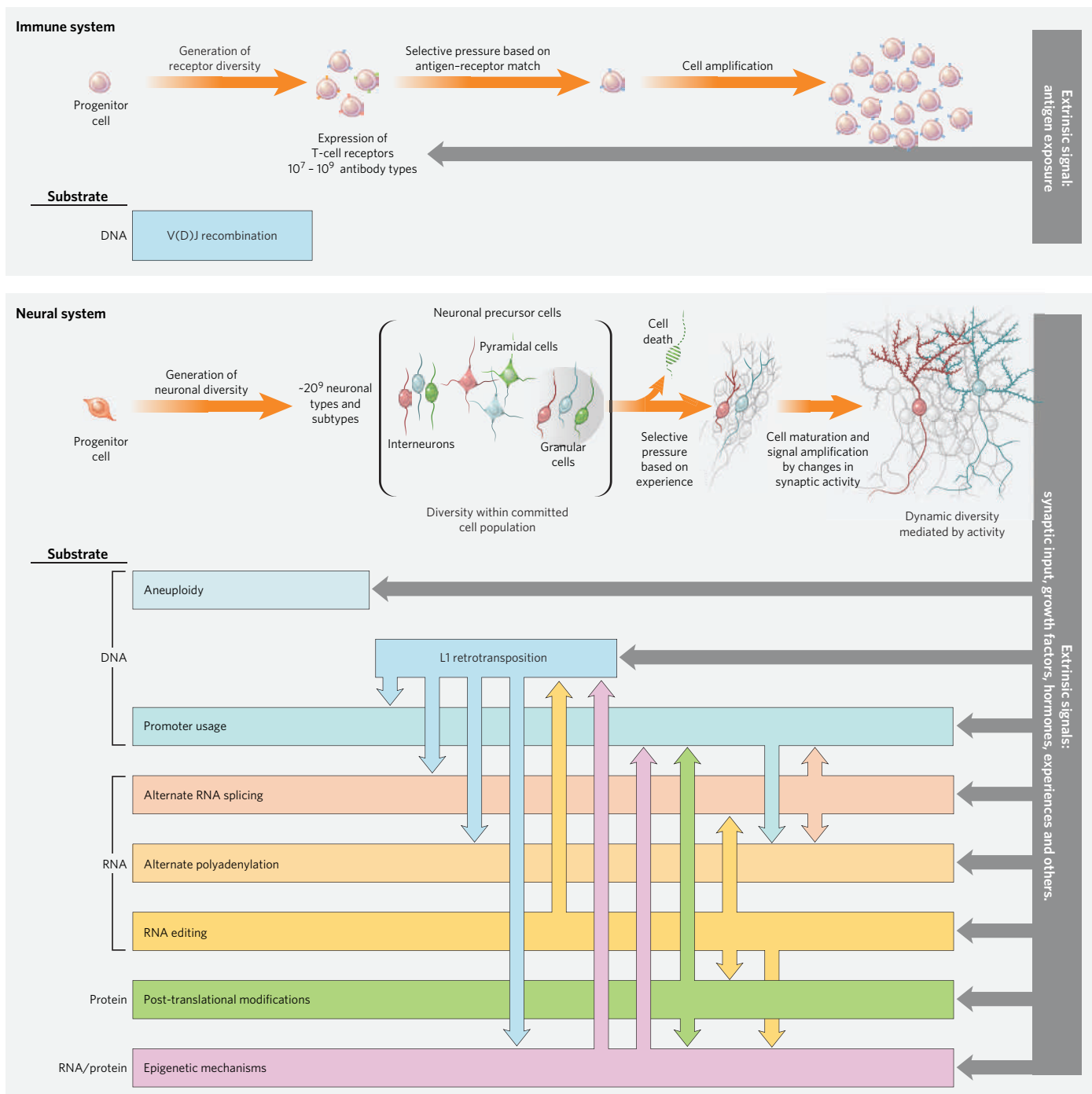


Figure 1 | Generation of somatic variability in the immune and neural systems. In the immune system, vast numbers of antibodies are generated by the V(D)J recombination system. In the nervous system, neuronal diversity is twice as high as the cellular diversity produced by the immune system. There is no single intrinsic mechanism that can explain such diversity, but rather a combination of several systems acting in different substrates (DNA, RNA or proteins) might be involved in neuronal differentiation. Finally, a dynamic diversity is generated by experience, correctly tuning neuronal wiring and generating diversity within subtypes of neuron. Contrary to the immune system, extrinsic factors can act directly on the intrinsic mechanisms of neuronal diversity and not only on selected cells. Moreover, intrinsic events that occur early during differentiation might only contribute to diversity in a more mature brain.

Aneuploidy

Aneuploidy is the loss or gain of chromosomes to produce a numerical deviation from multiples of the haploid chromosomal complement. Such chromosomal alteration has obvious consequences on cellular physiology, leading to well-documented diseases, such as Down's syndrome. Occasional somatic aneuploidy has frequently been associated with cell death²⁰ or cancer²¹. The original observation that aneuploid neural progenitor cells can divide and differentiate *in vitro* was attributed to a clonal tissue-culture artefact²². Chromosomal variation in neurons and glia of the developing and adult mammalian nervous system was

mainly detected by fluorescence *in situ* hybridization (FISH) in brain tissues of rodents and humans^{23,24}. From studies in mice, it is estimated that approximately 33% of neural progenitor cells display chromosomal aneuploidy that encompasses both loss and gain of chromosomes. However, the FISH technique presents potential artefactual problems that might interfere with sample analysis²⁵. Moreover, the overall percentage and complete chromosomal complement of aneuploid cells, hypoploidy, hyperploidy and several chromosomal combinations remain to be determined. Mature aneuploid neurons were described as being functionally active and integrated into brain circuitry, suggesting that a

network composed of intermixed euploid and aneuploid neurons might produce unique signalling properties, owing to its exceptional genetic-expression profile²⁶. Even the quantitative change of a single gene can produce major changes in cellular signalling²⁷, so the effects associated with loss or gain of approximately 1,000 gene copies per chromosome should produce even more drastic alterations in a cell's physiology and, consequently, in the neuronal network. However, the role of non-neuronal aneuploid (that is, glial) cell types is less obvious. No tissue-specific factor has been described that contributes to aneuploidy in the nervous system, and it seems that these cells arise, in part, via a chromosome mis-segregation mechanism during mitosis²⁸ (Fig. 2).

LINE-1 retrotransposition

The finding that LINE-1 retrotransposons (L1s) can have an effect on neuronal genomes challenges the dogma that neurons are genetically homogeneous units²⁹. L1s are among the most ancient and successful elements in eukaryotic genomes, comprising approximately 20% of mammalian genomes^{30–32}. Active human L1s are about 6 kb long, harbouring an internal promoter and encoding two open reading frames (ORFs) — an RNA chaperone protein, ORF1, and an endonuclease and reverse transcriptase (RT) protein, ORF2 — usually ending with a short tail that is rich in adenine bases. Upon translation, the L1 RNA assembles with its own encoded proteins³³ and moves back to the nucleus, where the endonuclease makes a single-stranded nick and the RT uses the nicked DNA to prime reverse transcription from the 3' end of the L1 RNA. Moreover, the L1-encoded proteins can function *in trans* to mobilize non-autonomous retrotransposons (such as Alu elements) and cellular messenger RNAs, generating processed pseudogenes^{33–36}. L1s can threaten the structure and regulate the expression of the genome in different ways, such as insertional mutagenesis^{37,38}. L1s are probably silenced in neural stem cells due to SOX2-mediated transcription repression²⁹. Downregulation of Sox2 accompanies chromatin modifications, such as DNA demethylation and histone acetylation, which, in turn, might trigger neuronal differentiation (Fig. 3). Despite the low number of L1 insertions characterized, the sequence data from target insertional sites in rat neuroblasts were often close to, or inside, neuron-associated genes, indicating that the integration process might be regulated²⁹. Some of these target genes included those for an olfactory receptor, ion-channel-associated genes and a cadherin receptor²⁹. It is challenging to estimate precisely the potential activity of endogenous L1s in neuroblasts. Most RNA molecules can be subject to retrotransposition if hijacked by L1 machinery^{33–36}. Thus, each developing neuron can potentially carry L1-mediated events and, if part of the resultant insertions occurs in genes expressed during neuronal development, it is possible that brain development could be significantly affected by L1s.

Promoter usage and multiple start sites

Promoters and enhancers are *cis*-acting elements that control gene transcription via a complex arrangement of protein–DNA and protein–protein interactions³⁹. For decades, the concept that these regulatory mechanisms control only the quantity, and not the identity, of their corresponding transcripts dominated our conception of gene expression. Many eukaryotic genes contain multiple promoters, each subjected to different regulatory factors present in a cell at a certain moment⁴⁰. By definition, each promoter determines a different start site and first exon, and, consequently, a different transcript. Frequently, transcripts arising from genes with multiple promoters only differ in their 5' non-coding regions, sharing the same ORFs (Fig. 4). Minimal promoter sequences or enhancers can be used to selectively drive transcripts in a spatially and temporally specific manner³⁹. Multiple transcriptional start sites are a common attribute of the glutamate receptor ion-channel genes. The expression of the various glutamate receptor genes is under developmental control and often responds to environmental stimuli. In several cases, the promoter regions include overlapping motifs for the transcription factors Sp1 and GSG near the major initiation sites, and a silencer element to guide expression in neurons⁴¹ (Fig. 4).

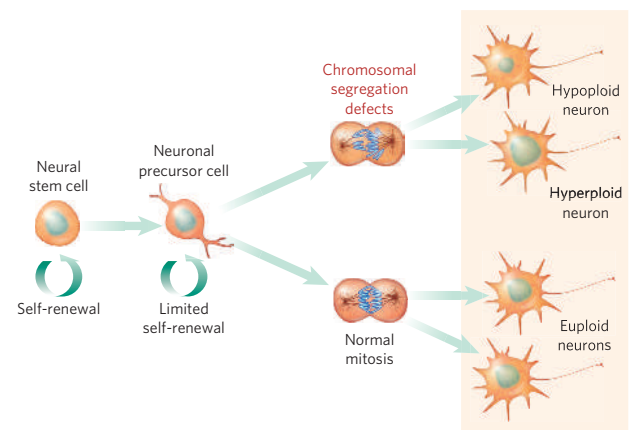


Figure 2 | Aneuploidy as a mechanism to alter gene expression in the CNS. Chromosomal segregation defects during mitosis can generate aneuploid neurons and astrocytes²⁸, resulting in a euploid population intermixed with an aneuploid population of cells.

A better understanding of the regulation of genes with multiple start sites will require the identification of the conditions under which each promoter becomes activated or repressed. The brain-derived neurotrophic factor (*BDNF*) gene contains a complex pattern of regulation, including the use of a variety of promoters. Four promoters have been mapped upstream of the 5' exons; these are differentially controlled by the Ca^{2+} signals evoked via *N*-methyl-D-aspartate (NMDA) glutamate receptors or L-type voltage-dependent calcium channels (L-VDCCs)⁴². The diversity of regulatory sequences could allow different constellations of transcription factors that are present in discrete parts of the brain to regulate BDNF in ways that produce differential patterns of isoform expression. Studies on the effect of acute cocaine exposure reveal promoter-specific upregulation of specific BDNF transcripts in the striatum and frontal cortex, whereas exposure to kainic acid can stimulate (by up to 50-fold) other BDNF transcripts in the hippocampus, implying the existence of different forms of synaptic plasticity in the two systems^{43,44}.

Alternative RNA splicing

Precursor RNAs made by eukaryotic RNA polymerase II (pol II) undergo a series of post-transcriptional processing events to produce mature mRNAs, which are then exported to the cytoplasm. Physiological alterations in RNA processing can serve as important control points of gene expression in a tissue or during development⁴⁵. Alternative RNA splicing is a versatile mechanism for generating a large repertoire of functionally unique proteins and occurs particularly frequently in the nervous system⁴⁶ within neuronal genes, such as those for neurotransmitter receptors, ion channels, synaptic surface receptors, axon-guidance receptors and transcription factors⁴⁷. The importance of alternative splicing in the nervous system became evident following the association of aberrantly spliced mRNAs with several neurological diseases⁴⁸. A striking example of complex alternative splicing is the *Drosophila* axon-guidance receptor (*DSCAM*), which is a homologue of the human Down's syndrome cell-adhesion molecule. The alternatively spliced exons each encode immunoglobulin half-domains that combine to create a receptor with as many as 38,016 different protein variants — double the predicted number of genes in the *Drosophila* genome⁴⁹. The mammalian homologue of *Dscam* is not subject to similar levels of alternative splicing; however, other mammalian genes implicated in cell-surface recognition and synapse formation, such as the neuroligins, can generate numerous isoforms⁵⁰. Cadherin-related neural receptors (CNRs or protocadherins) are encoded in an arrangement that is intriguingly reminiscent of the antibody genes, with 52 variable exons in humans, each encoding an entire extracellular and transmembrane region, grouped in three tandem arrays upstream of a single set of constant exons encoding the cytoplasmic region⁵¹.

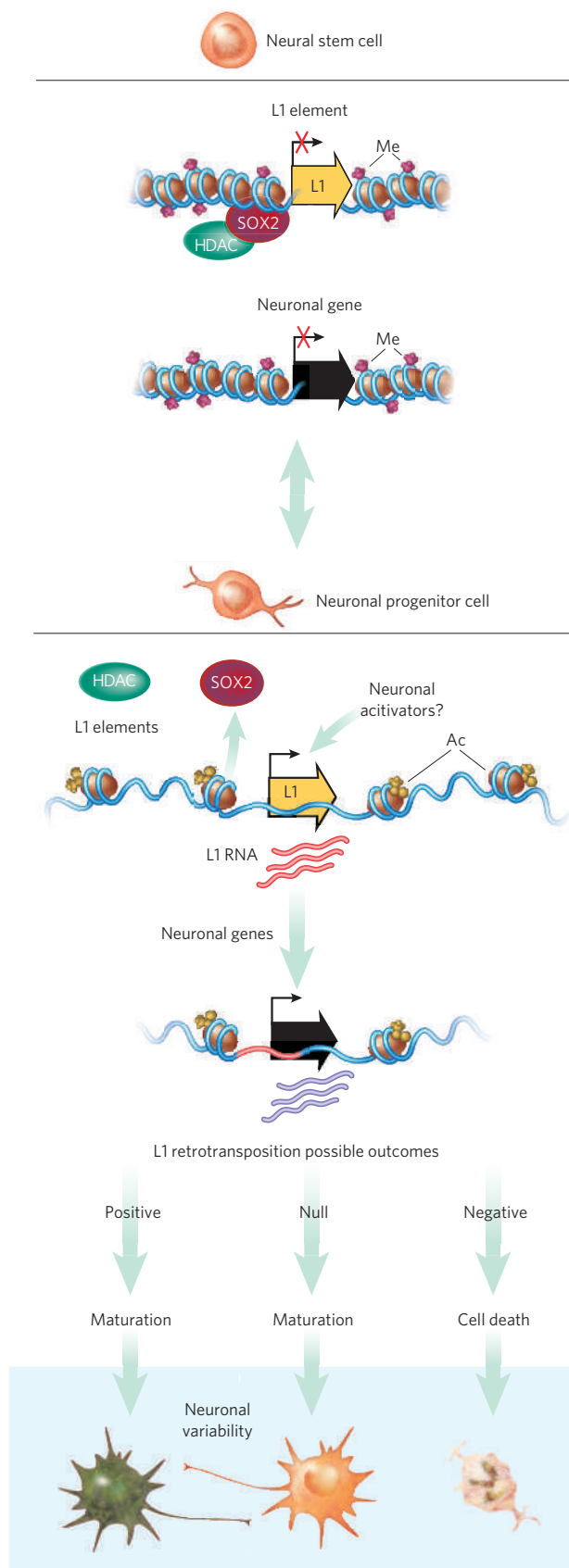


Figure 3 | A model for generation of neuronal diversity by L1 retrotransposition. During neuronal differentiation, L1 transcription is activated by chromatin modifications and release of SOX2, allowing subsequent retrotransposition into neuronal genes²⁹. The resulting retrotransposition events can alter gene expression, which can, in turn, influence the phenotype of the resulting cell. HDAC, histone deacetylase.

Promoter choice and splice-site selection seem to be responsible for the expression of individual protocadherin mRNAs⁵². Alternative splicing commonly regulates molecules that mediate cell excitation. Changes in the splicing of these transcripts in response to electrical activity represent an attractive additional mechanism for inducing long-term changes in excitability. Indeed, splicing of NMDA receptor 1 transcripts can be altered in response to various stimuli^{53,54}.

Differences in alternative splicing from individual cells in the brain have been examined using single-cell reverse transcription (RT)–polymerase chain reaction (PCR) combined with patch-clamp recordings⁵⁵. These experiments revealed that differences in splicing from cell to cell are apparent even within a defined population of neurons. Moreover, neurons in different regions of the brain display splicing differences that are consistent with their function, suggesting a sophisticated regulatory mechanism⁵⁵. Mechanisms for alternative splicing control in neurons can include cell excitation, splice-site choice, RNA-binding proteins, splicing enhancement, exon definition and neuron-specific RNA factors, such as Nova, Hu and neural polypyrimidine tract-binding proteins^{48,56}. The recent demonstration that the methyl-CpG-binding protein 2 (MeCP2), which has been implicated in Rett syndrome, can act as a splicing factor, linking transcription splicing to epigenetics, indicates that other factors are likely to exist⁵⁷.

Alternative polyadenylation

mRNAs synthesized in the nucleus generally contain a 3' poly(A) tail; the rare exceptions are the transcripts of replication-dependent histone genes. A functional polyadenylation signal is required for transcription termination by RNA pol II and transport from nucleus to cytoplasm, where the poly(A) tail on the message stabilizes the mRNA against degradation. Poly(A) sites can directly influence the amount of cytoplasmic RNA produced from a transcript; therefore, alterations in polyadenylation efficiency can have a profound effect on the amount and nature of a gene product. Alternative polyadenylation can be a direct consequence of tandem poly(A) signals within a single 3' untranslated region (UTR), of competition between promoter-proximal polyadenylation and splicing sites, or of the presence of alternative 3'-terminal exons that can be skipped in some circumstances. All of these strategies can produce proteins differing at their carboxyl termini, depending on which processing pathway is followed (Fig. 5). Many genes have been characterized as having an alternative polyadenylation site at the 3' end of their mRNAs, which can be preferentially used depending upon the cellular environment⁵⁸. In the nervous system, several examples of alternative polyadenylation have been described. *MeCP2* encodes four different transcripts distinguished by the length of the 3' UTR. The long (10 kb) mRNA form is highly expressed in a subpopulation of cells in the adult brain, in contrast to the shorter transcripts found in the lungs and liver⁵⁹. Moreover, the developmental dynamic of *MeCP2* in the brain suggests a potential role of the 3' UTR in the regulation of protein synthesis in response to age-specific requirements of *MeCP2* function⁶⁰.

Alternative polyadenylation seems to be more abundant in the nervous system than in other tissues⁶¹. The study of pre-mRNA cleavage and polyadenylation has revealed several factors required for accurate RNA processing, including cleavage and polyadenylation-specificity factor (CPSF), cleavage-stimulatory factor (CstF), poly(A) polymerase (PAP), the cleavage factors Im and IIm, and the poly(A)-binding proteins (PABPs). These proteins interact to form a complex on the precursor RNA prior to the cleavage and polyadenylation reactions⁶². Determination of the differential expression levels of these factors in the nervous system throughout development will provide a better understanding of the differential use of multiple poly(A) sites in complex transcription units.

RNA editing

Similar to RNA splicing, RNA editing alters the sequence of RNA from the encoded DNA by changing as few as one or two nucleotides. Alterations include converting one base to another, removing one nucleotide and substituting another, deleting encoded residues and inserting

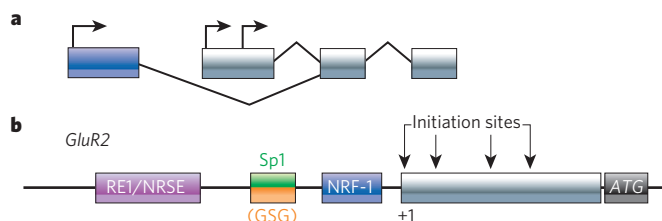


Figure 4 | Alternative start sites. **a**, Alternative promoter regions, 5'-splice sites or start sites can alter the length of the mRNA. **b**, Schematic of the proximal promoter region for the *GluR2* gene. The neuron-restrictive silencer factor REST/NRSF can repress the *GluR2* promoter via the neuron-restrictive silencer element RE1/NRSE. Apart from the silencer, Sp1 and nuclear respiratory factor-1 (NRF-1), regulatory elements within 60 base pairs of the +1 initiation site are required for strong promoter activity in cortical neurons. A potential GSG motif is also present, overlapping with the Sp1 binding site.

non-template nucleotides. Two types of RNA editing have been found in nuclear-encoded mRNAs, both of which involve deamination of encoded nucleotides: the first involves the deamination of cytidine (C) to create uridine (U), and the second involves the deamination of adenosine (A) to create inosine (I). Both types of editing can cause changes at the amino-acid level and frame shifts in mRNAs, creating *de novo* ORFs. They can also affect RNA secondary structure, such as in transfer RNAs and ribosomal RNAs, and can modify start and stop codons. Other processes that can be affected include RNA splicing, transport and stability; however, not all editing events have obvious effects, as some codon changes are silent or fall within non-coding regions of mRNA. Editing can be regulated in a developmental, environmental, hormonal or tissue-specific manner⁶³, and is most abundant in mRNAs in the nervous system⁶⁴. The mammalian editing enzymes, adenosine deaminases acting on RNA (ADARs), are widely expressed in several tissues; however, all known edited RNAs occur only in the CNS. The importance of such enzymes was revealed through gene knockouts in different systems. Although ADAR knockouts in flies and worms lead to behavioural abnormalities^{65,66}, knockouts in mammals have shown them to be essential for life⁶⁷.

Several subunits of glutamate receptor channels contain a glutamine residue that is subjected to A-to-I editing of the CAG codon into an arginine residue (Q to R in the single-letter amino-acid code) that changes a key residue within the ion pore of the channel. The unedited Q form of the GluR-B subunit renders α -amino-3-hydroxy-5-methyl-4-isoxazole propionic-acid (AMPA) receptors more permeable to calcium ions. This substitution can have several functional consequences, such as voltage-independent gating, decreased single-channel conductance and permeability to divalent cations, as the new amino acid affects the hairpin loop that forms the narrow constriction of the channel⁶⁸. Specific Q/R site editing in the GluR6 transcript was demonstrated to be relevant for receptor-independent long-term potentiation at a point at which the medial perforant path synapses on the DG granule cells, increasing the vulnerability to kainite-induced seizures⁶⁹. These findings indicate a clear role for RNA editing of GluR6 in synaptic plasticity, circuit excitability and seizure susceptibility. Furthermore, mRNA glutamate receptor editing is crucial for neuronal survival, and reduction of RNA editing might be a direct cause of selective motor-neuron death in amyotrophic lateral sclerosis patients⁷⁰. The serotonin receptor gene can generate 18 different complementary DNA (cDNA) sequences and 12 different predicted protein isoforms, owing to partial editing at the five A-to-I sites in a region involved in coupling to G proteins⁷¹. Moreover, some edited isoforms are differently expressed in distinct brain regions and have different affinities for some antipsychotic drugs, suggesting that predicted protein products are likely to be functionally important^{71,72}. Thus, the levels of editing in this receptor might be influenced by factors that alter levels of serotonin in the brain. Altered editing levels have been observed in schizophrenic patients, and might affect patients with Huntington's disease and Alzheimer's disease⁷³. Depressed suicide victims were also reported to have altered transcript-editing levels in the prefrontal cortex⁷⁴.

Post-translational modifications

Post-translational modifications of cellular proteins can regulate protein stability, activity and localization. Protein modification by covalent attachment of radicals to target proteins, such as ubiquitination, sumoylation or phosphorylation, is rapid and reversible, being a potent mechanism for the spatiotemporal control of protein activity, especially at the synapse level⁷⁵. The beauty of protein-modification choreography can be appreciated in the control of synaptic differentiation by the transcription myocyte enhancer factor 2 (MEF2). Phosphorylation of a serine residue in MEF2 prevents activation of its target genes, whereas dephosphorylation can be induced by calcium influx, triggering MEF2-dependent transcription in hippocampal neurons. In cerebellar granule neurons, the phosphorylated isoform of MEF2A can switch from activator to repressor by sumoylation, suggesting that phosphorylated and sumoylated MEF2A represses transcription of target genes, promoting synaptic differentiation. This sumoylation switch might underlie the differential effect of MEF2 on synapse number in cerebellar and hippocampal neurons^{76,77}. Post-translational regulation can also induce epigenetic modifications through chromatin modification, regulating expression in a set of genes (see below).

Epigenetic mechanisms

Epigenetic modifications, including DNA methylation and histone modification (such as acetylation and methylation), can drive a specific, reversible and heritable pattern of gene expression on differentiating cells, without affecting the DNA sequence. Such mechanisms allow changes in chromatin structure, coordinating the activation/repression of gene arrays at precise times during development. Although several examples of neuronal plasticity exist, the study of epigenetic modifications in cellular diversity is in its infancy^{78,79}. The combinatorial methylation of specific lysine residues at the amino-terminal tails of the histones H3 and H4 is increasingly accepted as a molecular imprint involved in the regulation of gene expression in eukaryotes⁸⁰. Interestingly, levels of dimethylated and trimethylated histone H3 at lysine 4 showed significant differences between human glutamate promoters in different brain regions and during development, reinforcing the idea of histone methylation as a transcriptional regulator in the CNS⁸¹. DNA methylation is another type of epigenetic modification that mediates gene silencing by inhibiting transcription-factor binding to promoter regions or by binding methyl-CpG-binding proteins, such as MeCP2, leading to transcriptional repression. One of the well-known functions of DNA methylation is to suppress transcriptional activity of endogenous retro-elements, including L1s. There is evidence that endogenous retroviral transcripts are expressed in the nervous system of patients with schizophrenia and other complex psychotic diseases; however, the role of all these transcripts is obscure⁸²⁻⁸⁷. Theoretically, such an abundance of endogenous transcripts could represent precursor RNAs to several

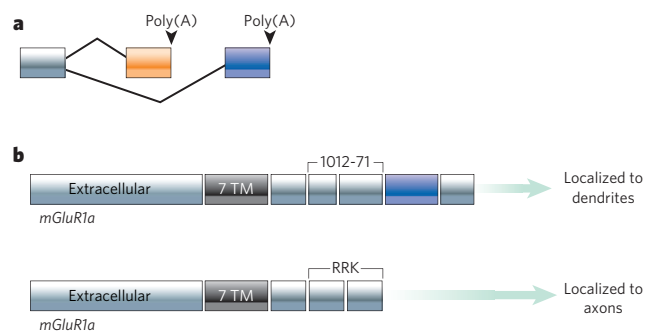


Figure 5 | Alternative poly(A) sites. **a**, Choosing from several distinct polyadenylation sites, transcripts with 3'-differential exons can be generated. **b**, Truncation of the GluR1 carboxy-terminal by alternative splicing redirects the protein product from neuronal dendrites (dominant signal 1012-71) to axons (RRK motif)⁹⁶. 7 TM, seven transmembrane-spanning region.

non-coding small RNAs with potential regulatory functions in the cell. Alternatively, these endogenous retroviruses might be more active in the brains of affected individuals and contribute to the phenotype.

Non-protein-coding RNAs can also cause post-transcriptional modification and might influence epigenetic modifications. They seem to be mostly expressed in the brain, suggesting that individual gene expression or clusters of genes in the nervous system can be highly affected by the action of these versatile molecules^{88,89}. MicroRNAs (21–25 nucleotides) are derived from longer RNA precursors, processed in the nucleus and subsequently exported to the cytoplasm, where they associate with the RNA-induced silencing complex (RISC) that directs the microRNAs to their targets for post-transcriptional repression⁹⁰. Computational algorithms have suggested that microRNAs have the ability to simultaneously regulate many targets, providing a means of coordinated control for concerted gene action⁹¹. Despite the scarcity of functional examples, data regarding predicted targets, expression patterns and overexpression studies suggest that microRNAs are attractive key players for differential gene expression in many neuronal types.

Concluding remarks

Understanding what produces neuronal diversification has been a long-standing challenge for neuroscientists. More than a century ago, Camillo Golgi published a technique for the silver staining of isolated neurons in samples of highly dense cells in a brain slice. The Golgi technique was important because it allowed Ramon y Cajal to realize the incredible morphological diversity of neurons, directly reflecting their synaptic connections. Nowadays, the production of such neuronal diversity from a relatively homogeneous population of neural stem cells in culture has great promise for the treatment of several neurological diseases (see page 1094).

The evolution of several genetic strategies in eukaryotes greatly expanded the number of functionally distinct proteins that could be produced from a given gene pool. However, are all these strategies required or even adequate to generate such complex neural networks? The answer is probably no. Almost certainly, other mechanisms remain to be uncovered and understood. Emerging studies of non-coding RNAs have provided us with tantalizing examples of regulatory molecules⁸⁸. For instance, small double-stranded modulatory RNAs have been proposed to regulate the generation of neurons from adult neural stem cells by working as a transcriptional co-activator for a large set of neuronal genes⁹². The combined use of several systems, along with new neuron-specific mechanisms that provide extra adaptability to an individual process (for instance, L1 insertions creating new splicing sites or polyadenylation signals^{93–95}) and extrinsic signals, is likely to be responsible for generating the large spectrum of protein activities that supports complex cognitive brain functions, such as consciousness and plasticity. ■

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Stem cells for the treatment of neurological disorders

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Many common neurological disorders, such as Parkinson's disease, stroke and multiple sclerosis, are caused by a loss of neurons and glial cells. In recent years, neurons and glia have been generated successfully from stem cells in culture, fuelling efforts to develop stem-cell-based transplantation therapies for human patients. More recently, efforts have been extended to stimulating the formation and preventing the death of neurons and glial cells produced by endogenous stem cells within the adult central nervous system. The next step is to translate these exciting advances from the laboratory into clinically useful therapies.

It is hoped that stem cells will provide an inexhaustible source of neurons and glia for therapies aimed at cell replacement or neuroprotection in disorders affecting the brain and spinal cord (Fig. 1). Embryonic stem (ES) cells, and stem cells from the fetal or adult central nervous system (CNS) or other tissues might all be suitable for this purpose, but human cells are probably needed for clinical application. The development of stem-cell therapies will require a detailed knowledge of disease pathology and how specific cell types in various areas of the CNS are affected. Different cell types and neuroprotective molecules will be needed depending on the disorder. In the case of some disorders, gains can probably be induced only with transplanted cells generated from stem cells *in vitro*, whereas in other conditions the stimulation of endogenous CNS stem cells may be beneficial.

Here, we consider several neurological disorders for which stem-cell-based therapy has raised particular interest. We describe the ways in which stem cells might be used to treat these conditions, discussing the prospects for and problems of translating laboratory findings into clinically useful therapies.

Parkinson's disease

The pathological hallmark of Parkinson's disease (PD) is a gradual loss of nigrostriatal dopamine-containing neurons, but degeneration also occurs in systems of non-dopaminergic neurons. The main symptoms are rigidity, poverty of movement (bradykinesia), tremor and postural instability. Current therapies centre on the oral administration of L-dopa and dopamine receptor agonists, and on deep-brain stimulation in the subthalamic nucleus. These treatments are effective for some symptoms, but are associated with side effects and do not stop the progression of the disease. To be clinically competitive, a stem-cell-based therapy must lead to long-lasting, significant improvement in mobility, ameliorate currently intractable symptoms, or counteract disease progression.

Clinical trials of the transplantation of human fetal dopaminergic neurons have shown that cell replacement can produce major, long-lasting improvement in some patients¹. So it is promising that cells with properties of dopaminergic neurons have been generated *in vitro* from stem cells of various sources, such as ES cells and stem cells isolated from bone marrow and fetal brain^{1–3}. However, whether any of these protocols could induce functional recovery of therapeutic value is unclear, because there has not yet been a clear demonstration that once transplanted in animals

with experimental PD, the neurons generated *in vitro* can efficiently reinnervate the striatum, release dopamine *in vivo*, and give rise to considerable recovery from deficits resembling human symptoms¹. To make stem-cell therapy work for PD, dopaminergic neurons with the characteristics of substantia nigra neurons⁴ must be produced in large numbers. For dopaminergic neurons generated from human ES cells⁵, survival after

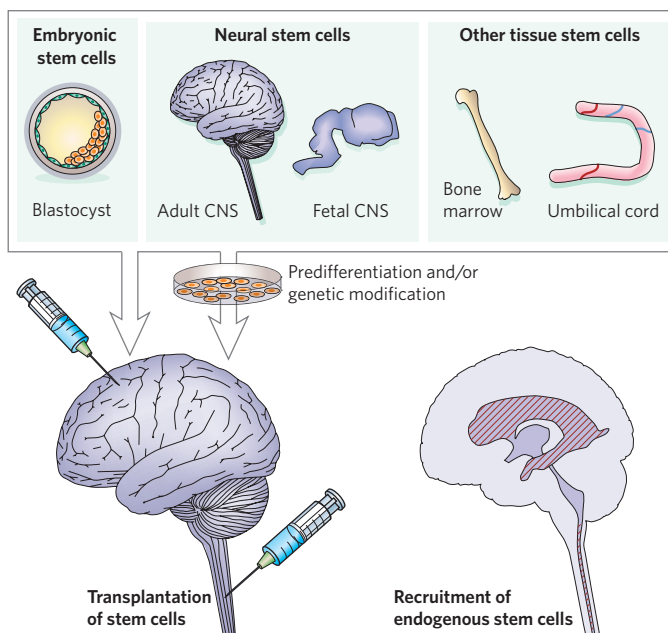


Figure 1 | Application of stem cells for neurological disorders. Stem cells would be isolated and transplanted to the diseased brain and spinal cord, either directly or after predifferentiation/genetic modification in culture to form specific types of neuron and glial cell, or cells producing neuroprotective molecules. In strategies relying on stimulation of the patient's own repair mechanisms, endogenous stem cells would be recruited to areas of the adult brain and spinal cord affected by disease, where they would produce new neurons and glia (neurogenic and gliogenic areas along lateral ventricle and central canal are shown in hatched red). Stem cells could provide clinical benefits by neuronal replacement, remyelination and neuroprotection.

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transplantation in animal models has been poor and needs to be markedly increased before clinical application. Because some patients will need implants in several areas of the brain⁶, optimum recovery will require a tailor-made grafting procedure based on preoperative imaging. It will also be necessary to develop strategies that hinder disease progression. One possible approach to prevent the death of existing neurons could be to transplant human stem cells engineered to express neuroprotective molecules such as glial-cell-line-derived neurotrophic factor (GDNF)⁷.

Stroke

Stroke is caused by blockage of a cerebral artery, leading to focal ischaemia, loss of neurons and glial cells, and motor, sensory or cognitive impairments. No effective treatment to promote recovery exists, so a therapy that produced even minor improvement would be valuable. Transplanted cells from different sources, such as fetal brain, neuroepithelial or teratocarcinoma cell lines, bone marrow and umbilical cord, have yielded some improvement in animals and, in one clinical trial, in humans affected with stroke¹. In most cases, the grafts have acted by providing trophic factors that enhance cell survival and function¹. However, for stem-cell therapy to be of major clinical value, human cells should be able to replace dead neurons, remyelinate axons and repair damaged neural circuitries.

As a first step towards this goal, human fetal neural stem (NS) cells were transplanted into the brains of stroke-damaged rats, resulting in the migration of new neurons towards the ischaemic lesion⁸. Other studies showed that monkey ES-cell-derived progenitors transplanted into the brains of mice after stroke differentiated into various types of neuron and glial cell, re-established connections with target areas⁹, and led to improved motor function¹⁰. The therapeutic efficacy of such strategies could be improved further by genetically modifying the stem cells: for example, by overexpressing an anti-apoptotic gene¹¹.

Interestingly, the stroke-damaged adult rodent brain has some capacity for neuronal replacement from its own NS cells. For several months after a stroke, NS cells can generate new striatal neurons that migrate to the site of damage^{1,12}. It is now important to establish whether endogenous neurogenesis can contribute to functional recovery after stroke, and whether it occurs in humans. And, because the regeneration of cortical neurons will be the basis for functional improvement in most stroke-damaged brains, we will also need to know whether the adult brain's own NS cells can be triggered to produce cortical neurons. Effective therapies will depend on strategies to increase the survival of the new neurons and to enhance their incorporation into reorganizing neural circuitries.

Huntington's disease

Huntington's disease (HD) is a fatal, intractable disorder that is characterized by chorea (excessive spontaneous movements) and progressive dementia. It is caused by the death of projection neurons in the striatum. Stem-cell therapy aims to restore or preserve brain function by replacing and protecting striatal neurons — a strategy that might be insufficient because patients also suffer progressive neocortical degeneration. In animal models of HD, cell replacement using grafts of fetal striatal neurons promotes functional recovery, and some evidence from clinical trials indicates that this can also occur in patients¹. By contrast, stem-cell-based approaches are still in their infancy, and the reconstruction of striatal neural circuitry has not been shown in animals. However, human NS cells implanted into the brains of rats were recently found to reduce motor impairments in experimental HD through trophic mechanisms^{13,14}. At this time, using stem cells for the delivery of trophic factors and neuroprotection to prevent disease progression seems a more achievable clinical goal in HD than neuronal replacement.

Amyotrophic lateral sclerosis

In amyotrophic lateral sclerosis (ALS), dysfunction and degeneration of motor neurons occur not only in the spinal cord (lower motor neurons) but also in the cerebral cortex and brainstem (upper motor neurons). Muscle weakness progresses rapidly and death occurs within a few

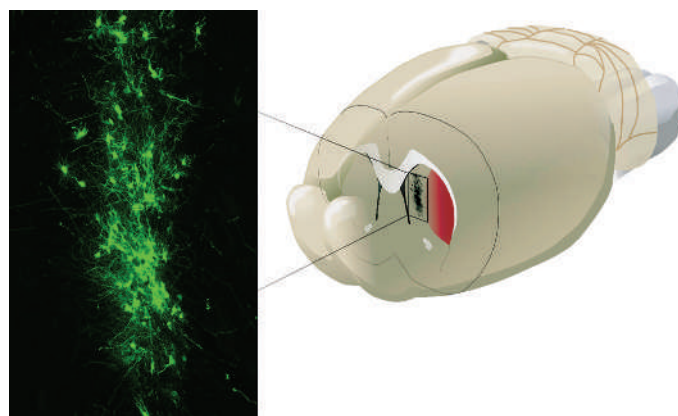


Figure 2 | Transplantation of stem cells into injured brain. Human fetal NS cells labelled with green fluorescent protein survive for at least 1 month and differentiate into cells morphologically resembling neurons after grafting in close proximity to the stroke-damaged rat striatum (red area).

years. There is no effective treatment. A stem-cell therapy must restore or preserve the function of both upper and lower motor neurons, and new neurons must become integrated into existing neural circuitries. Recent reports have shown that it is possible to generate lower motor neurons *in vitro* from stem cells of various sources, including ES cells and those from the fetal CNS^{1,15,16}. Mouse ES-cell-derived motor neurons establish functional synapses with muscle fibres *in vitro*^{17,18} and extend axons to ventral roots after transplantation into adult rats¹⁷. But whether these neurons can integrate into existing neural circuitries and restore motor function has not been established.

Whereas neuronal replacement in ALS patients seems a distant goal, using stem cells to prevent motor neurons from dying is a more realistic and shorter-term clinical approach. This prospect is supported by studies showing that human embryonic germ cells delivered into the cerebrospinal fluid of rats with motor neuron injury can migrate into the spinal cord and induce motor recovery, probably through neuroprotection¹⁹. The efficacy of this approach could be improved by genetically modifying the stem cells to secrete molecules that promote motor neuron survival. For instance, a recent study showed that human cortical progenitors that were engineered to express GDNF survived implantation into the spinal cords of ALS rats and released the neurotrophic factor²⁰.

Alzheimer's disease

Alzheimer's disease (AD) is characterized by neuronal and synaptic loss throughout the brain, involving the basal forebrain cholinergic system, amygdala, hippocampus and several cortical areas. Patients' memory and cognitive performance is progressively impaired; they develop dementia; and are likely to die prematurely. Current therapies, such as treatment with acetylcholinesterase inhibitors to enhance cholinergic function, give only partial and temporary alleviation of symptoms.

The pathological changes seen in AD offer an extremely problematic situation for cell replacement. Given the widespread and progressive damage in the brains of patients with AD, it is unlikely that the mechanisms for instructing transplanted NS cells to differentiate into new neurons will be intact. In theory, cognitive decline caused by the degeneration of basal forebrain cholinergic neurons could be prevented by transplanting cholinergic neurons generated from NS cells *in vitro*. But to provide long-lasting symptomatic benefit, this approach would require the existence of intact target cells within the patient's brain, and these are highly likely to be damaged.

However, because stem cells can be genetically modified and have migratory capacity after transplantation, they could be used for the delivery of factors that can modify the course of the disease. In support of this approach, basal forebrain grafts of fibroblasts that produce nerve growth factor (NGF) — which counteracts cholinergic neuronal death, stimulates cell function and improves memory in animal models — have been of some benefit in patients with AD²¹.

Multiple sclerosis

Multiple sclerosis (MS) is caused by the inflammation-induced destruction of the myelin sheath that surrounds axons, leading to conduction deficits and a variety of neurological symptoms and, in some patients, major disability. Axonal loss as a consequence of acute inflammation or chronic demyelination is an important cause of functional deterioration. Immunomodulatory and immunosuppressive treatments are only partially effective.

Myelin-producing oligodendrocyte progenitor cells (OPCs) are abundant in the adult human brain²². Spontaneous remyelination occurs to varying degrees in the early stages of MS, and OPCs are also present in chronic demyelinated MS lesions. An important area of research is that focused on finding ways to enhance remyelination from these cells, and identifying the factors that lead to a failure of cells to produce myelin in the first place. To this end, Back *et al.*²³ recently showed that astrocyte-derived hyaluronan accumulated in demyelinated lesions from MS patients and prevented the maturation of endogenous OPCs.

The transplantation of remyelinating cells represents another approach for treating myelin loss in MS. Human adult²² and ES-cell-derived²⁴ OPCs have been shown to myelinate dysmyelinated mouse brain and spinal cord after transplantation. However, a major concern is that the inflammatory environment could destroy the grafted OPCs and inhibit their maturation. Immunosuppressive and anti-inflammatory treatments might therefore be necessary. Another problem is that the demyelinated MS lesions are distributed across multiple locations throughout the CNS. An effective therapy will require the implanted OPCs to migrate to these sites. Interestingly, after systemic administration in mice, NS cells migrated to inflammatory demyelinating lesions, where some became OPCs and remyelinated axons²⁵. Most cells remained undifferentiated and suppressed proinflammatory mechanisms²⁶.

Spinal cord lesions

Spinal cord injuries interrupt ascending and descending axonal pathways, and cause a loss of neurons and glia, inflammation and demyelination. The lesions lead to a loss of movement, sensation and autonomic control below the site of injury. There is no cure, and the most common current treatment — high-dose methylprednisolone — is of questionable value.

The transplantation of stem cells into injured spinal cord can lead to functional benefits^{27,28}, mainly through trophic factor secretion or the remyelination of spared axons. A recent study showed that human NS cells implanted into damaged mouse spinal cord generated new neurons and oligodendrocytes, leading to locomotor recovery²⁹. However, there are risks of side effects unless NS-cell differentiation after transplantation is controlled. Astrocytic differentiation and aberrant axonal sprouting after NS-cell implantation into injured rat spinal cord can cause hypersensitivity to stimuli that are not normally painful³⁰.

Perhaps the most realistic short-term clinical goal is to use stem cells for remyelination, which probably occurs to some degree after lesions from endogenous OPCs³¹. One study reported that after NS-cell implantation into injured spinal cord in rats, there was a good correlation between the number of graft-derived oligodendrocytes, the amount of myelin, and the extent of functional recovery³⁰. Another study reported that transplanted oligodendrocytes from human ES cells could myelinate the injured rodent spinal cord and improve motor function³².

Perspectives

It would be premature to launch clinical trials to use stem cells to treat neurological disorders. However, steady progress supports the hope that stem-cell-based therapies to restore and preserve function in the brain and spinal cord can be developed. For each disease, it is now possible to develop a road map that defines the necessary scientific and clinical advances required for stem cells to reach the clinic. Before we apply stem-cell therapies to patients, we must be able to control the proliferation and differentiation of stem cells into specific cellular phenotypes and to prevent tumour formation. Furthermore, the efficacy of stem cells and their mechanisms of action should be demonstrated in animal models with pathology and symptomatology resembling the human disease.

Even so, it may be difficult to translate data obtained in animals to humans because of species differences in the degree of neuronal plasticity and an incomplete knowledge of disease mechanisms. We must understand how to influence the pathological tissue environment, including inflammatory and immune reactions, to allow efficient repair. Finally, we must remember that however exciting the neurobiological mechanisms might be, the clinical usefulness of stem cells will be determined by their ability to provide patients with neurological disorders with safe, long-lasting and substantial improvements in quality of life. ■

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Potential of stem-cell-based therapies for heart disease

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The use of stem cells to generate replacement cells for damaged heart muscle, valves, vessels and conduction cells holds great potential. Recent identification of multipotent progenitor cells in the heart and improved understanding of developmental processes relevant to pluripotent embryonic stem cells may facilitate the generation of specific types of cell that can be used to treat human heart disease. Secreted factors from circulating progenitor cells that localize to sites of damage may also be useful for tissue protection or neovascularization. The exciting discoveries in basic science will require rigorous testing in animal models to determine those most worthy of future clinical trials.

Rarely has anything so energized scientists and the lay public alike as the enormous potential of stem-cell biology to treat human disease. The ability to mobilize endogenous progenitor cells in organs or to introduce and differentiate exogenous stem cells for tissue repair could have an impact on many diseases, including those affecting the brain, skeletal muscle, pancreas and heart. Regenerative therapies could be particularly beneficial for heart disease — the number one killer in adults and the leading non-infectious cause of death in children¹. Cardiomyocytes do not seem to enter the cell cycle after birth, and consequently the heart has almost no regenerative capacity after injury. The long-held dogma has been that the heart cells with which you are born are the ones with which you die.

However, exciting new findings in the past 5 years have caused us to re-evaluate the potential of protective or regenerative cardiac therapies. Like the brain, the heart seems to have reservoirs of progenitor cells that may not be sufficient to replace the acute loss of a large number of cells, but may be able to replace a slow apoptotic loss of cells over a lifetime. Here, we examine the current basic and clinical science that is forming the foundation of future approaches to cardiac regeneration.

Cardiac and ES-cell-derived progenitor cells

Throughout the myocardium, pools of cardiac progenitor cells (CPCs) may participate in the continual replacement of apoptotic cardiomyocytes at a low basal level. Unlike terminally differentiated cardiac cells, CPCs are small cells that do not express cardiac markers and that can self-renew and proliferate. Several seemingly different but overlapping populations of progenitor cells (such as Sca-1- (ref. 2), c-Kit- (ref. 3) and Abcg2- (ref. 4) expressing side populations) can be induced to activate cardiomyocyte-specific genes *in vitro*; however, this effect has been also observed in mesenchymal stem cells, which do not fully differentiate into functional heart cells⁵. In addition, Sca1- or c-Kit-expressing cells may differentiate into cardiomyocytes *in vivo*, contributing to repair of the damaged heart after acute myocardial infarction, but their potential is limited, in part, by the small number of endogenous CPCs. Attempts to mobilize and to expand endogenous progenitor cells by introducing growth factors hold promise but remain controversial⁶. It is likely that the activity of endogenous CPCs will have to be augmented, through knowledge of the mechanisms of normal

progenitor expansion and determination during embryonic development, before these cells will contribute substantially in the extreme setting of infarcted hearts.

Because embryonic stem (ES) and progenitor cells resemble early fetal cells that are adopting discrete lineages, elucidating the early developmental events of cardiogenesis has been instructive for understanding and manipulating CPCs. Pluripotent stem cells maintained through transcriptional regulators (such as NANOG, OCT4 and SOX2; ref. 7) are directed to differentiate into the mesoderm lineage by key transcription factors, including MESP and the T-box protein brachyury^{8,9} (Fig. 1). Subsequent determination of mesoderm progenitors mimics the embryonic developmental potential of two distinct fields of cells that give rise to the heart. Often referred to as the first and second heart fields, cells in these regions express unique markers of progenitor cells¹⁰. For example, the LIM-domain-containing transcription factor islet1 (ISL1) is involved in the differentiation of second heart field cells¹¹, whereas the homeodomain-containing transcription factor NKX2.5 is a marker of both heart fields¹⁰. Most interestingly, remnant second heart field cells may not only be able to differentiate into many cell types, but also persist in the postnatal heart¹² (Fig. 1). The pool of potential CPCs might be involved in continual maintenance of the heart by differentiating into several types of cardiac cell, including muscle, conduction and vascular cells, although the precise lineage potential of distinct subtypes remains to be determined (Fig. 2). It will be important to identify specific markers of the primary heart field to locate progenitor cells postnatally and even into adulthood.

Developmental markers of the primary and secondary heart fields may be useful in enriching ES cells for CPCs. When allowed to grow in clusters called 'embryoid bodies', mouse and human ES cells can differentiate into many lineages, including beating cardiomyocytes. Numerous approaches to increase the number of CPCs in this system have been devised, including the addition of retinoic acid or dimethyl sulphoxide, and the inhibition of BMP signalling¹³. Modulation of WNT signalling may also be useful¹⁴. In addition to key signalling and transcriptional events that may direct the cardiac lineage, the discovery of microRNAs that are muscle-specific (for example, miR-1 and miR-133) and involved in differentiation of CPCs in the embryo^{15,16} raises the interesting possibility that microRNAs could be useful in initiating or sustaining the

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cardiogenic programme. Finally, improvements in chemical libraries and high-throughput screens make it possible to screen for small molecules that could regulate the proliferation or differentiation of CPCs¹⁷.

In summary, numerous studies are underway to isolate and modify the behaviour of CPCs. Such pursuits are essential because the direct introduction of undifferentiated ES cells into the heart results in the formation of teratomas¹⁸. The different lines of investigation and approach described above provide hope that we may be able to regulate the commitment, proliferation and differentiation of ES or non-ES cells into cardiomyocytes, and to harness these cells for therapeutic purposes.

Other types of progenitor cell

Advances in understanding the basic biology of stem cells have been balanced by mixed and controversial results from translational and clinical studies. Early studies in mice suggested that bone-marrow-derived mesenchymal stem cells (BMSCs) could differentiate into cardiomyocytes¹⁹, and thus the introduction of BMSCs after myocardial infarction might induce the repair of damaged myocardium. More recent studies with genetically marked cells indicate that BMSCs do not transdifferentiate into cardiomyocytes^{20–22}. It remains possible that BMSCs do confer some beneficial effects, possibly by secreting paracrine factors that are cardioprotective or angiogenic²³.

On the basis of early mouse work suggesting myogenesis, numerous clinical trials with autologous BMSCs were begun in individuals with acute myocardial infarction or ischaemic myocardium²⁴. Early anecdotal reports suggested some benefit, but subsequent randomized trials yielded mixed results. Several small studies reported a statistically significant increase in cardiac pumping ability and coronary perfusion, and a decrease in infarct size. At least one larger recent trial, however, has not shown a clinical benefit²⁵. Similarly, a randomized trial based on earlier mouse data²⁶, in which granulocyte colony-stimulating factor was used to mobilize endogenous BMSCs after infarction, did not show improvement in cardiac function²⁷.

If BMSCs do confer physiological benefit, then they do so by an unknown mechanism, raising concern for ongoing or pending clinical trials, many of which initially assumed that BMSCs can differentiate into new cardiomyocytes. Although the focus has now shifted to understanding the potential non-cell-autonomous effects of BMSCs on hypoxic myocardium, much remains to be learned. Secreted angiogenic factors and/or activation of pathways that promote cell survival might protect and rescue hypoxic myocardium, thereby limiting damage to tissue and improving cardiac function²³. If paracrine factors are the key agents, isolating and delivering such factors at high concentrations or engineering BMSCs to secrete larger amounts could result in more significant protection²⁸. Interestingly, thymosin β 4, which is secreted in very large quantities by BMSCs^{28,29}, is cardioprotective after acute myocardial infarction³⁰ and induces angiogenesis in mice^{31,32}. Future large-animal and clinical trials of thymosin β 4 and other secreted factors hold promise and may obviate the need for cell-based therapy for the at-risk hypoxic myocardium.

The road ahead

Future research on the clinical applications of stem cell biology for human heart disease will be based on advances in understanding cell lineage decisions and the regulation of pluripotency and differentiation in cardiac and vascular cells. Approaches for regenerating damaged muscle and for treating children who lack specific subsets of cardiac lineages will face many hurdles. The ability not only to guide and expand stem cells into the cardiac lineage but also to repress alternative fates will be crucial to avoid differentiation into cell types that may be harmful to cardiac homeostasis. Methods for safe delivery, migration and proper integration of stem cells will need to be perfected to avoid complications and abnormal electrical coupling that could lead to arrhythmias, as has been experienced with introduction of skeletal muscle into the myocardium³³. Finally, it will be essential to solve the immunological issues surrounding rejection if non-autologous sources of stem cells

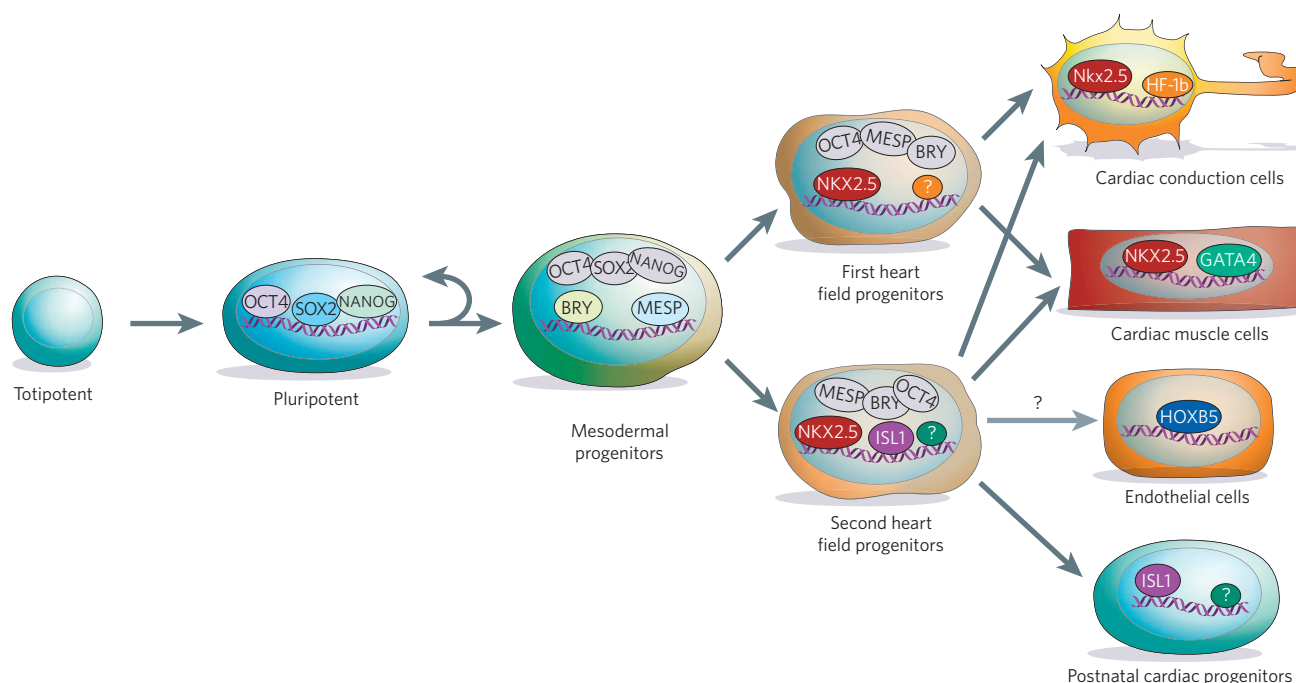


Figure 1 | Differentiation of embryonic cells into the cardiac lineage. Transcriptional regulation of early embryonic cells by factors such as NANOG, OCT4 and SOX2 maintains pluripotency. Decreased activity of pluripotency factors is accompanied by increased activity of lineage-specific transcriptional activators such as brachyury (BRY) and MESP in the mesoderm lineage. Mesodermal cells that begin to differentiate into cardiac cells segregate into two distinct populations of cardiac progenitor cells (CPCs), marked by the transcription factor NKX2.5. Those CPCs that also express the transcription factor ISL1 are similar to cells derived from the embryonic second heart field, whereas those without ISL1 expression may be more like first heart field cells. These progenitors may be able to differentiate into several types of cardiac cell, and ISL1-expressing cells uniquely give rise to niches of postnatal CPCs. Some transcription factors involved in lineage decisions are indicated. GATA4, GATA-binding protein 4; HF-1b, trans-acting transcription factor 4; HOXB5, homeobox B5.

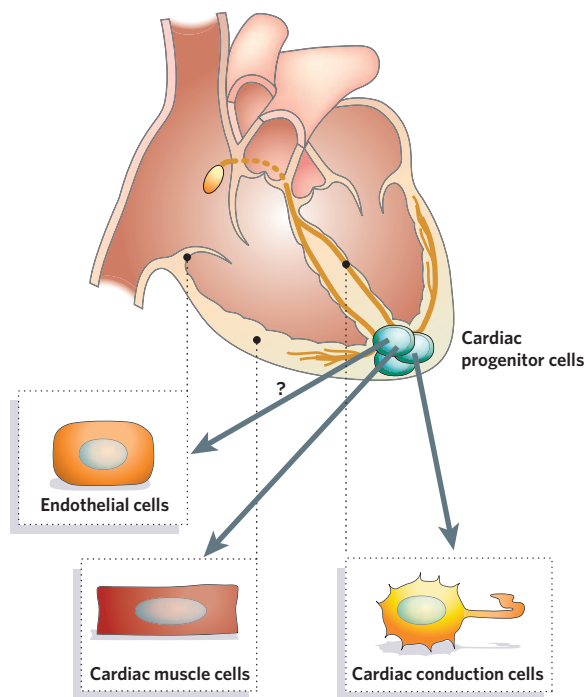


Figure 2 | Potential of postnatal cardiac progenitor cells (CPCs). Niches of CPCs in the postnatal heart may have the potential to differentiate into endothelial cells for vessel formation, cardiac muscle cells for contractility, and cardiac conduction cells for coordinated electrical activity of the heart, although precise lineage potentials of distinct subsets remain to be determined.

are used. In this respect, technologies to develop individual-specific stem-cell lines through somatic-cell nuclear transfer³⁴ or cell fusion³⁵ may allow engineered stem cells containing the individual's own genetic material to be used both for treatment and for studies of pharmacological efficacy.

Although there are clearly many obstacles to overcome, it is significant that a roadmap of the derivation and use of stem cells for human heart disease is now conceivable. The coming years will undoubtedly bring new developments and technologies that will require rigorous scientific evaluation and prudent judgment, balancing healthy scepticism with eagerness driven by sobering clinical needs. ■

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Stem-cell therapies for blood diseases

Claudio Bordignon¹

For decades, transplantation of haematopoietic stem cells — either unmodified, or genetically modified to correct genetic disorders — has been used to treat disorders of the blood and immune systems. The present challenge is to reduce the risk of such transplants and increase the number of patients who can safely access this treatment. In developing countries, such ‘one-shot’ treatments are highly desirable because chronic treatments are difficult to sustain. To make these therapies more accessible and effective it will be important to improve clinical protocols and gene-delivery vectors, and to gain a deeper understanding of stem cells.

Since the 1950s and 1960s, bone-marrow transplants, in the form of intravenous infusions of whole marrow, have been used to treat patients with acute leukaemia, bone-marrow aplasia¹ and congenital immune deficiencies². These clinical treatments led to basic studies to identify, isolate and characterize the rare stem cells (haematopoietic stem cells, or HS cells) that populate the cellular component of the marrow and peripheral blood³, and to understand their therapeutic potential. This work has been seminal in the field of stem-cell research, and has provided reagents and models for other tissue-specific and organ-specific stem cells. It was recognized by the 1990 Nobel Prize awarded to E. Donnall Thomas⁴.

Despite these successes, the wider therapeutic application of HS-cell transplantation to blood disease is limited by the lack of suitable donors, the high rate of mortality associated with the procedure and the recurrence of the underlying diseases. Here, I discuss the limitations of current HS-cell-transplant therapies, and review efforts to improve their therapeutic potential and availability to treat a wider spectrum of patients suffering from diseases of the blood.

HS cells in health and disease

Bone marrow was, for many years, transplanted as an unfractionated tissue, until scientists discovered which cellular components were responsible for the engraftment of the donor haematopoietic and immune systems in marrow-ablated patients⁴. These cells, the HS cells, have turned out to be among the most active in the human body. As the cellular and subcellular components of the blood (such as white cells, red cells and platelets) have life spans of only days or months, HS cells must continually produce new ones. Under normal physiological conditions, quiescent blood stem cells are interspersed with differentiated cells within the marrow of flat and short bones. However, under conditions of stress, such as massive bleeds or acute bacterial infections, HS cells respond rapidly by accelerating their proliferation, differentiation and migration from the marrow to circulate throughout the body.

HS cells are so potent that, even after an injury has wiped out most of the peripheral blood and marrow cells, it takes only a small number to fully restore a functional haematopoietic system. In mouse models, less than one hundred highly purified HS cells can fully reconstitute the blood system of recipient animals after the bone marrow has been completely ablated by radiation³. In experiments in mice, serial transplantation of HS cells — in which they are isolated from the marrow of the recipient and used to reconstitute a second marrow-ablated recipient — can be repeated several times before stress on the original HS cells leads to incomplete reconstitution.

The powerful growth and expansion properties of HS cells make them attractive candidates for a range of clinical applications. There is also

great progress in methods for treating patients with growth hormones in order to expand HS cells and the HS-cell population, and mobilize them from the bone marrow into peripheral blood, from which they can be readily isolated in relatively high numbers (see page 1075). Therapies for HS-cell mobilization have the advantages of avoiding the complex and invasive procedure of bone-marrow explant under general anaesthesia, while providing higher yields of purified HS cells. Such improvements have even made it possible to perform transplants from partially incompatible donors, and have contributed to HS-cell transplantation becoming the treatment of choice for a number of genetic and acquired disorders^{5–9} (Table 1).

Complexities of HS-cell transplantation

Successful HS-cell transplantation relies on a complex interplay of factors, including an intact bone-marrow niche, stem-cell competition, reaction of donor immune cells to diseased or malignant cells of the recipient, and efficacy of treatments designed to eliminate diseased cells prior to transplant. Successful engraftment of HS cells has been shown to require an intact marrow environment (or niche), which provides the signals for homing, seeding and expansion of the transplanted stem cells (for a more complete description of the role of the stem-cell niche, see page 1075). Studies of bone-marrow transplants used to treat leukaemia have shown that immune recognition and elimination of leukaemic cells by the donor immune system have central roles in patient outcome — a condition called graft-versus-leukaemia. Thus, leukaemic recurrence in bone-marrow transplantation can depend on the degree of identity of genes that regulate tissue compatibility between donor and recipient, with the highest rate of recurrence in genetically identical twins¹⁰.

The most confounding limitation of bone-marrow and HS-cell transplantation is the availability of appropriate donors. The ideal donors are healthy histocompatible family members, who are available for only about one-third of patients. Despite efforts to create international registries of unrelated donors and HS-cell repositories from umbilical cord blood, a substantial proportion (up to 50%) of patients have no access to potentially life-saving transplants owing to a lack of suitable donors.

In recent years, technologies for purifying and transplanting HS cells have made it possible to perform transplants from half-matched family donors, who are available for almost all patients (the parents and children of any individual are genetically half-matched)¹¹. Unfortunately, the hopes that HS-cell transplantation could provide a solution have been frustrated by unacceptably high rates of transplant-related mortality in recipients of such half-matched HS-cell transplants. Whereas highly purified HS cells from half-matched donors can reconstitute a functional bone marrow and nearly all cellular components of the blood

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Table 1 Status of HS-cell therapies for genetic blood disorders			
	Form of treatment		
	Autologous*	Allogeneic†	Gene therapy or delayed lymphocyte infusion‡
Inborn errors			
SCID	–	X	Clinical
Haemoglobinopathies	–	X	Experimental
Inborn errors of the metabolism	–	X	Experimental
Fanconi anaemia	–	X	Experimental
Acquired diseases			
Acute leukaemias	–	X	X
Myelodysplasia	–	X	X
Lymphomas	X	X	?
Multiple myeloma	X	X	?
Myeloproliferative disorders	–	X	X
Paroxysmal nocturnal haemoglobinuria	–	X	–
Aplastic anaemia	–	X	–

*Stem-cell transplantation from the same individual. †Stem-cell transplantation from normal healthy donors. ‡Gene therapy relates to inborn errors, whereas delayed lymphocyte infusion refers to adoptive immunotherapy by donor lymphocytes and is related to acquired diseases. X denotes a treatment that is currently in clinical use for a disease. Clinical denotes ongoing clinical trials. Experimental denotes a preclinical or initial clinical application. Dashes indicate therapies for which there is no clinical rationale or clear clinical benefit. Question marks indicate that a clinical benefit has not yet been formally proved. SCID, severe combined immunodeficiency.

in a marrow-ablated patient, they fail to provide the lymphocytes necessary for rapid and complete immune reconstitution. Thus, the patient remains unprotected against potentially lethal viruses and other infectious agents, and has a high risk of dying from infection. Attempts to overcome this problem by combining stem-cell transplantation with infusions of unmanipulated lymphocytes from the donor have been thwarted by the tendency of donor immune cells to react against the patient's organs and tissues. Such reactions are often lethal because of the high degree of mismatch between the donor and patient. Strategies for immune protection of the recipient by the use of defined subsets of lymphocytes are being developed and tested in mouse models (reviewed in ref. 12). Alternatively, genetic manipulation of donor lymphocytes to

insert a suicide-gene system has been shown to control this complication in patients¹³. It is hoped that such strategies will eventually allow HS-cell transplants for many more patients with malignant diseases of the haematopoietic system and lethal genetic disorders.

HS-cell transplantation for the developing world

Developing countries of the Far and Middle East and Africa have large numbers (believed to be tens of thousands) of patients affected by lethal red-cell disorders. One such disease is thalassaemia major, which is a genetic disorder that affects haemoglobin. In Westernized countries, it is treated by packed red-cell transfusions from selected blood donors, which are administered periodically throughout the lifetime of the patient; these are combined with chronic drug therapy for preventing overload of the iron present in the red-cell transfusions. In most underdeveloped countries, however, there are no national blood-donor programmes, and thalassaemic patients die in childhood. Thus, purified HS cells from family donors could potentially provide an affordable therapy where transfusion programmes are unavailable. In this situation, HS-cell transplantation could not only offer a technologically advanced treatment with the best chance of curing the disease, but could also be used to treat relatively large numbers of patients. It is true that HS-cell transplantation is still an expensive procedure that carries a variable, but significant, risk of mortality. In the future, however, it is hoped that by simplifying HS-cell isolation and implementing less-toxic preparative regimens for patients, the expense and mortality of transplants will be reduced. Currently, most HS-cell transplantation for Middle Eastern thalassaemia patients is performed in Western countries with dedicated programmes (Fig. 1), such as that of Fondazione IME, an international health organization that has the principal responsibility of carrying forward the International Thalassaemia Project. If more international collaborations such as this could be established, the lives of thousands of young patients could be saved by such one-shot therapies, which might provide a permanent cure rather than life-long, expensive and poorly tolerated treatments.

HS-cell gene therapy

Gene therapy offers an alternative to stem-cell transplantation from healthy donors for the treatment of thalassaemia and certain other genetic diseases of the blood. In gene-therapy approaches, a patient's

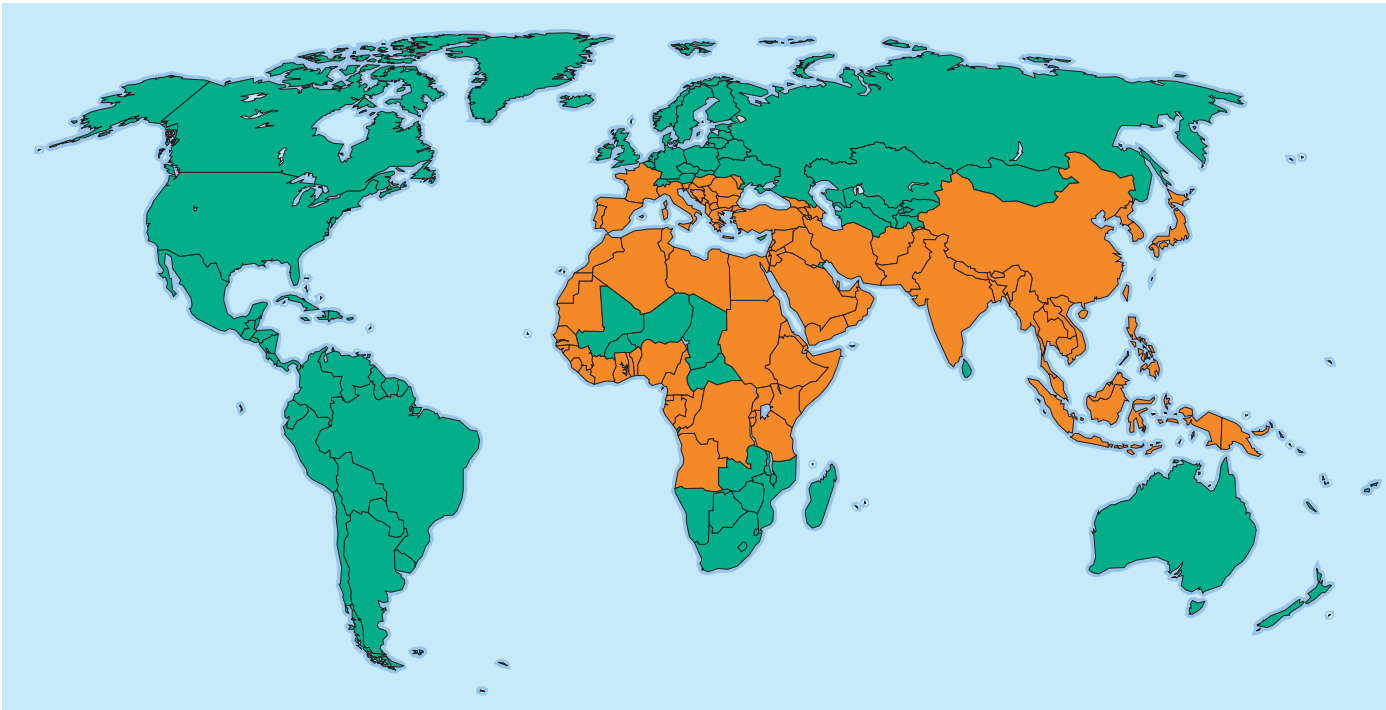


Figure 1 | Large-scale HS-cell therapy across the world. Countries potentially involved (orange) in clinical application of large-scale HS-cell therapy to treat genetic blood disorders.

own HS cells are isolated from bone marrow or blood, genetically engineered to express a normal copy of the gene responsible for the disease and then transplanted back into the patient. Since the early 1990s, this type of gene therapy has been successfully used to treat severe combined immunodeficiencies (SCIDs), using viral vectors to carry a healthy gene into the genome of the patient's HS cells¹⁴. Patients affected by the X-linked variant¹⁵, the adenosine deaminase (ADA)-deficient variant¹⁶ and chronic granulomatous disease¹⁷ seem to be permanently cured of their defects using this strategy.

Unfortunately, despite these clinical successes, HS-cell gene therapy for genetic diseases has not progressed at the expected pace. One reason is the difficulty involved in appropriately regulating expression of the transferred genes, which is a particular problem for the globin genes¹⁸. Another reason is related to the presentation of a severe adverse event in a clinical study of X-linked SCID¹⁹. In the clinical study, three patients, after obtaining optimal clinical correction of the disease, showed a progressive lymphoid proliferation that clinically resembled an acute leukaemia¹⁹. This adverse event could be directly linked to a specific clonal insertion of the viral vector used for gene transfer into the stem-cell host genome near a known oncogene²⁰. Despite the fact that this event seems to have several unique features associated with this specific trial (a similar study performed by a different group did not show any adverse event²¹), all stem-cell gene-therapy trials were subjected to intense scrutiny and were put on hold for varying periods of time in most countries. We expect that HS-cell gene therapy will progress at a faster pace now that safer and more efficient vectors are being produced and are entering clinical trials. In addition to congenital immunodeficiencies, a number of other genetic diseases of the blood might become candidates for such treatments. In particular, neurodegenerative disorders caused by the absence of specific enzymes, such as lysosomal enzymes, are good candidates for HS-cell gene therapy, as replacement enzymes can potentially be produced by cells generated from genetically engineered HS cells²².

HS-cell plasticity

In addition to the classical application of HS cells for treating blood diseases, there has been great interest in the idea of using them to treat diseases of other organs and tissues. Engraftment, and in some cases tissue repair, has been reported in a range of organs after systemic or local injection of HS cells or bone marrow. Initially it was thought that the HS cells responded to local stimuli by differentiating into mature cells of the recipient organ. Later, it was found that cell fusion was a major mechanism for stem-cell contribution to the regeneration of the treated organ. Although the jury is still out on the actual role of trans-differentiation in stem-cell-mediated tissue repair, cell fusion represents a more widely accepted general phenomenon, particularly for tissues possessing highly fusogenic cells, such as muscle²³ and liver²⁴. It is interesting that, in some cases, therapeutic benefit has been observed in the absence of significant levels of stem-cell engraftment. This apparent paradox can be explained by the ability of stem cells to produce stimulatory and regulatory cytokines²⁵ that stimulate tissue growth and repair.

Perspectives

It is remarkable that, through bone-marrow and HS-cell transplants, stem-cell therapies have brought about permanent cures for some (tens of thousands) patients suffering from blood disorders. Unfortunately, for other patients, there remains a 10–50% rate of transplant-related mortality and only a small chance of finding a suitable donor. Thus, transplant medicine seems far from offering an optimum cure for the majority of patients with blood disorders. There are efforts underway to develop therapies using alternative sources of stem cells, such as embryonic stem cells. However, because HS cells are relatively abundant and accessible, alternative sources might be less crucial for treating common blood disorders than for diseases of other organs and tissues. Thus, the most immediate improvements will probably be achieved by developing less-toxic drug regimens for use in preparing

the patient to accept the transplant and strategies for prompt immune reconstitution following transplant. Genetic engineering has already been successfully implemented in HS-cell therapy. However, more effective vectors and a better understanding of the consequences of vector integration¹⁷ are needed to expand the number of diseases that can be treated by this technology. Whereas genetic diseases are the most obvious candidates for HS-cell therapies, they might also be effective in treating acquired diseases, such as cancer²⁶ and AIDS, particularly for patients living in countries where the costs of life-long chronic treatments are unsustainable. ■

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Genetic evidence for complex speciation of humans and chimpanzees

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The genetic divergence time between two species varies substantially across the genome, conveying important information about the timing and process of speciation. Here we develop a framework for studying this variation and apply it to about 20 million base pairs of aligned sequence from humans, chimpanzees, gorillas and more distantly related primates. Human-chimpanzee genetic divergence varies from less than 84% to more than 147% of the average, a range of more than 4 million years. Our analysis also shows that human-chimpanzee speciation occurred less than 6.3 million years ago and probably more recently, conflicting with some interpretations of ancient fossils. Most strikingly, chromosome X shows an extremely young genetic divergence time, close to the genome minimum along nearly its entire length. These unexpected features would be explained if the human and chimpanzee lineages initially diverged, then later exchanged genes before separating permanently.

The genetic divergence between two species (the proportion of nucleotides differing between representative individuals of the two species) can be converted into a divergence time in terms of millions of years, provided that differences between genomes have accumulated at a constant rate as a result of new mutations^{1,2}. The average genetic divergence, τ_{genome} , is sometimes used to estimate the speciation time, τ_{species} . However, $\tau(x)$, the genetic divergence at any position x , fluctuates across the genome and is everywhere larger³ than τ_{species} (Fig. 1a, and Supplementary Note 1). Thus, its average τ_{genome} necessarily exceeds τ_{species} .

Inferring ancient speciation from genetic data

With the availability of large-scale sequencing, enough data can now be obtained to study not only the average τ_{genome} but the distribution $\tau(x)$. This should allow direct inferences about τ_{species} , which must be less than the minimum time divergence, and about variability in $\tau(x)$, which conveys information about the speciation process. Several issues must be considered in studying $\tau(x)$ for any pair of modern species. First, the genetic divergence should be corrected for local variation in the neutral mutation rates across the genome. This can be done by dividing the local divergence between two species by the divergence of one from an outgroup, for example macaque for the human-chimpanzee comparison. Second, any estimate, $\hat{\tau}(x)$, of local genetic divergence should be corrected for the effects of recurrent mutation; we do this using two independent methods. Third, variability of $\hat{\tau}(x)$ should be assessed by studying large enough subsets of the genome for the resulting estimates to be reliable.

Although chimpanzees are our closest relatives, there are many loci at which humans and gorillas (or chimpanzees and gorillas) are the most closely related^{4,5}; we estimate that this is the case over about 18–29% of the genome (Supplementary Note 2). In such places, the genetic divergence time of human and chimpanzee must precede gorilla speciation (Fig. 1b–d). Thus, humans and chimpanzees show large variation in $\tau(x)$, making them an excellent system in which to explore how taking into account the difference between τ_{genome} and τ_{species} can affect inferences about history.

Previous genetic analyses of great apes studied small data sets (the

largest was about 25 kilobases (kb))⁵ and suggested that the time since average genetic divergence of humans and chimpanzee genes is much greater than the time of speciation^{4,6–8}. However, they produced inconsistent estimates of ancient diversity owing to small data sizes, ignoring the effects of recurrent mutation⁹, and simplifying assumptions about the demography of ancient populations¹⁰.

Genome comparisons of five primates

To create a much larger data set, we generated shotgun sequence from the gorilla (about 115,000 fragments comprising about 87 megabases (Mb)), and compared it against the human¹¹ and chimpanzee genomes¹² and the unpublished sequence of orangutan and macaque (Methods). We overlapped the sequences, producing four-species human-chimpanzee-gorilla-macaque (HCGM) alignment (18.3 Mb) and five-species human-chimpanzee-gorilla-orangutan-macaque (HCGOM) alignment (9.3 Mb) (Supplementary Tables 1 and 2). We also studied 1.2 Mb (ref. 13) from contiguous regions of chromosomes 7 and X (Methods). Altogether, these data represent more than 800-fold more aligned bases than the largest data set previously available⁵, and enough data to compare chromosome X with the autosomes.

To analyse these data we identified all ‘divergent sites’, places at which two alternative alleles were observed across the aligned sequences of the species. We eliminated sites in hypermutable CpG dinucleotides¹¹, and those not flanked by at least one base of completely conserved sequence (our qualitative results are unaffected by these filters; Supplementary Table 3 and Supplementary Fig. 1). This produced 858,941 divergent sites for the HCGM shotgun data, 498,771 for HCGOM shotgun data, and 78,290 for the contiguous data.

We categorized the divergent sites according to how they partitioned the species (Table 1). In the four-species HCGM alignment, there are seven possible partitions: four in which one species differs from the other three (denoted H, C, G and M) and three in which two species differ from the other two (denoted HC, HG and CG). If all divergent sites were due to single historical mutations, the proportion of each class, $n_H:n_C:n_G:n_{HC}:n_{HG}:n_{CG}:n_M$, would be strictly

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proportional to relative ‘branch lengths’ of the genealogical tree—that is, the elapsed time on each branch averaged across the genome (assuming that mutations have accumulated at a constant rate over time^{1,2}) (Fig. 1b–d). However, the correspondence is not exact because some sites are due to more than one mutation⁹. Recurrent mutation has a particularly distorting effect on short branches. Because HG and CG sites occur rarely and can easily be generated by recurrent mutation, branch lengths t_{HG} and t_{CG} would be overestimated without this correction.

By using five-species alignment data (HCGOM), we were able to estimate the effect of recurrent mutation on branch length estimates, controlling for the biases it introduces. In particular, the HCGOM data revealed six classes of divergent site that could not be due to

single historical mutations (Table 1). An example is HO, which clusters together human and orangutan (Supplementary Table 4). We developed two methods that use the rates of these sites to correct the estimates of branch lengths for the impact of recurrent mutations (Supplementary Methods and Supplementary Note 3). Our analysis indicates that past studies that failed to correct for recurrent mutation^{5–8} obtained roughly twofold higher estimates of the rates of HG and CG sites (Supplementary Table 5). Thus, these studies were biased towards overestimating the proportion of the genome in which humans and chimpanzees are not most closely related^{5–7}.

Large variation in divergence time across genome

We used a straightforward approach to study variation in $\tau(x)$, avoiding the assumptions about the demography of ancient populations from previous studies^{5–8}. We selected subsets of the genome in which we proposed that the divergence would be different from the average. We then calculated the average value of $\hat{\tau}(x)$ across each subset and divided by $\hat{\tau}_{\text{genome}}$ to obtain the relative age, A , compared with the autosomal average (Supplementary Table 6).

We began by considering subsets of the genome consisting of the neighbourhoods of HC sites. The subsets $S_{\text{HC}}(d)$ were defined as all bases within a distance d of an HC site on an autosome (excluding the site itself, to obtain unbiased estimates of local genetic divergence). We expect that human and chimpanzee would be more closely related near HC sites. Analysis of the HCGOM shotgun data shows that relative age decreases with d (Fig. 2) and reaches a limit of $A \approx 0.862 \pm 0.009$ —that is, 86.2% of the average genetic divergence across the autosomes (Supplementary Note 4). Because the human–chimpanzee genome divergence time is thought to be about 7 Myr ago (refs 5, 10), this translates to a roughly 1-Myr reduction. The true difference must be even larger, because the average of $\tau(x)$ near HC sites must be greater than τ_{species} .

Second, we considered the neighbourhoods of HG or CG sites. The subsets $S_{\text{HG/CG}}(d)$ were defined as all bases within a distance d of either an HG or a CG site. We would expect that human and chimpanzee would be more distantly related near such sites. The relative age increases with d (Fig. 2) and reaches a limit of $A \approx 1.342 \pm 0.022$ (Supplementary Note 4). Near two HG or CG sites the increase is even greater: $A \approx 1.45 \pm 0.07$. This is primarily because two HG or CG sites close together are less likely to reflect recurrent mutations (comprising about 32% of HG and CG sites in the HCGOM data; Supplementary Table 5). When we use modelling to extrapolate the value of A near HG or CG sites in the absence of recurrent mutation, we infer that the true limit is even greater: $A \approx 1.47$ (Methods).

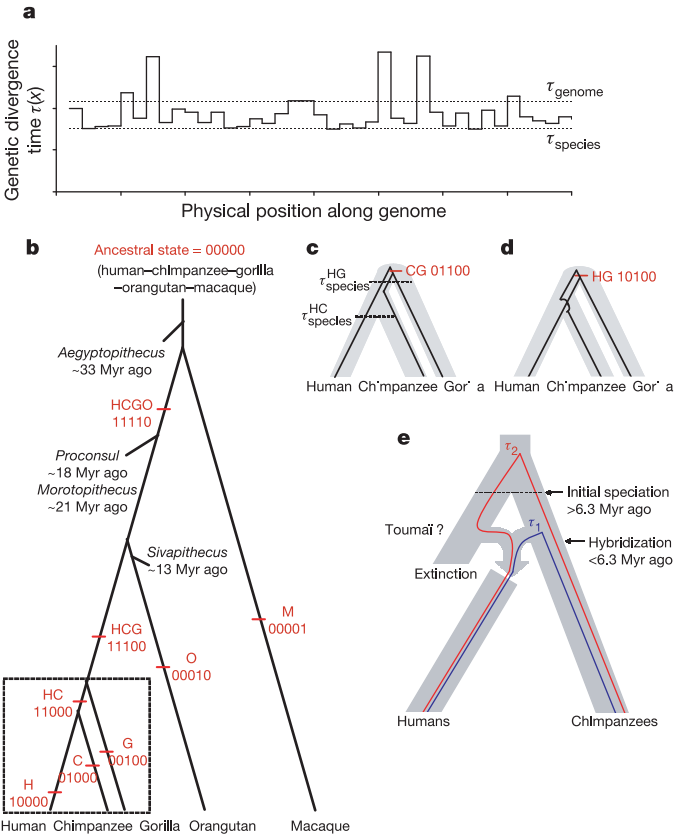


Figure 1 | Genetic relationships differ from species relationships. **a**, The genetic divergence time between two species, $\tau(x)$, varies across the genome and is always greater than or equal to the speciation time, τ_{species} , which is the time of last gene flow between the species’ ancestors. The average genetic divergence (τ_{genome}) thus always exceeds τ_{species} . **b**, The historical relationships of the species included in this study, along with relationships to various fossils (adapted from ref. 43). The relative lengths of the branches can be estimated from the data by the number of divergent sites of each type. **c**, **d**, Genealogical relationships are not always the same as the species relationships (grey), because humans and chimpanzees can sometimes share a common ancestor that is older than the gorilla speciation (greater than $\tau_{\text{species}}^{\text{HG}}$). For example, although humans and chimpanzees are most closely related in most sections of the genome, there are regions in which chimpanzees and gorilla are most closely related^{4–6} (producing ‘CG’ sites, **c**), or in which humans and gorilla are most closely related (‘HG’ sites, **d**). **e**, A revised model that could explain our data is that the first hominins became isolated from chimpanzee ancestors more than 6.3 Myr ago, but then hybridized back to the chimpanzee lineage. This could explain the great variation in divergence time across the genome, with humans and chimpanzees sharing a common ancestor around the time of hybridization in some regions (blue line) and before the initial speciation in others (red line). A model with chimpanzee ancestors as the hybrids is equally consistent with the data.

Table 1 | Main data sets in the study

Class	Pattern	Autosomes		X chromosome		
		Species Bases	HCGOM 8,899,720	HCGM 17,552,410	HCGOM 372,354	HCGM 747,260
n_H	10000		28,504	59,175	936	2,008
n_C	01000		28,495	59,844	944	1,935
n_G	00100		38,677	81,671	1,430	3,138
n_{HC}	11000		8,561	20,408	457	1,035
n_{HG}	10100		1,302	4,809	14	95
n_{CG}	01100		1,430	4,600	12	86
n_{HCG}	11100		41,928		1,493	
n_O	00010		82,670		3,086	
n_M	11110		244,270	596,939	9,621	23,198
n_{HO}	10010		412		9	
n_{CO}	01010		397		11	
n_{GO}	00110		764		22	
n_{HCO}	11010		1,347		56	
n_{HGO}	10110		989		30	
n_{CGO}	01110		872		32	

Each divergent site class is designated by a string of 0s and 1s, the bases seen in human–chimpanzee–gorilla–(orangutan)–macaque. The macaque allele is defined to have state ‘0’.

Large reduction in divergence time on chromosome X

Third, we considered the divergence along individual chromosomes, and especially chromosome X. The relative divergences for the autosomes are all close to the average (Fig. 3), but divergence is reduced along nearly the entire length of chromosome X (Fig. 3). A slightly reduced age for chromosome X is in fact expected from population genetic theory; the population size of chromosome X should be three-quarters of that of the autosomes, and thus the coalescent time should be three-quarters as large. Calculations would predict $A \approx 0.918\text{--}0.943$ (Supplementary Note 5), but the observed value is much younger: $A \approx 0.835 \pm 0.016$ (Supplementary Table 6).

To confirm the low divergence of chromosome X, we performed the same analysis for human–gorilla divergence and found no discrepancy between the expected $A \approx 0.932\text{--}0.958$ (Supplementary Note 5) and the observed $A \approx 0.977 \pm 0.028$ (Supplementary Table 6). This excludes the possibility that the reduced divergence in the human–chimpanzee comparison reflects a slowdown of the mutation rate on chromosome X in the apes, because this would affect the gorilla comparison as well. As an independent line of evidence, we note that if human–chimpanzee divergence on chromosome X is recent, we would not expect segments of human–gorilla or chimpanzee–gorilla clustering (Fig. 1c, d). In fact, the rate of HG and CG sites is one-quarter of the autosomal rate. The rate is slightly lower than would be expected if the sites were due entirely to recurrent mutation; the 95% credible interval for the proportion of HG and CG sites not due to recurrent mutation is 0–15% of the autosomal rate (Supplementary Note 6). The data are consistent with a complete absence of regions where humans and gorillas, or chimpanzees and gorillas, are most closely related.

The data point to an enormous decrease in genetic divergence for chromosome X in comparison with the autosomes. On the basis of $A \approx 0.835 \pm 0.016$ for chromosome X (Supplementary Table 6) and calibrating to an estimate of human–chimpanzee genome divergence

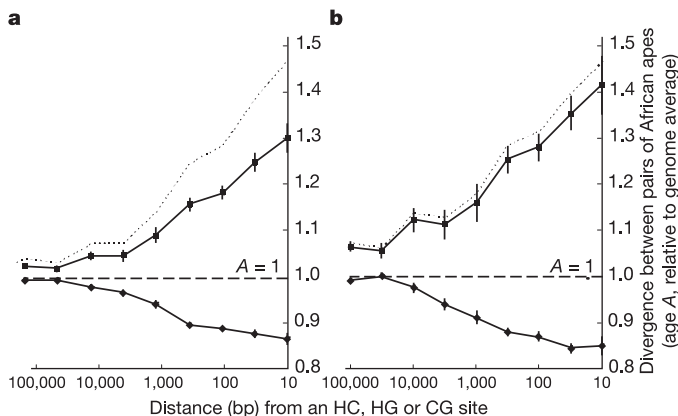


Figure 2 | Near a region of HG, CG or HC clustering, $\tau(x)$ deviates strikingly and significantly from the genome average. **a**, For the HCGM shotgun data, the human–chimpanzee divergence is less than 88% of the genome average near an HC site (upper solid line) and at least 124% of the genome average near an HG or CG site (lower solid line). **b**, For the HCGOM shotgun data, the observed proportions are 86% and 134% (Supplementary Note 4). The true range is certainly larger than shown in either graph because HG and CG sites resulting from recurrent mutation (which are more frequent for HCGM than HCGOM data) dilute the estimated increase in human–chimpanzee divergence near these sites. Correcting for this by using a modelling analysis fitted to the HCGOM shotgun data (Supplementary Table 11) indicates that the divergence near true HG and CG clusters might be about 147% of the genome average. A dotted line corresponding to this extrapolated divergence is shown. (No line is shown for HC because it is very similar to the unextrapolated line.) Each data point is obtained by averaging all bases within a window geometrically centred on distance d ($d/\sqrt{10}$ to $d\sqrt{10}$). Error bars here and in Fig. 3 give ± 1 standard error.

about 7 Myr ago^{2,5}, the average age difference between chromosome X and the autosomes must be about 1.2 Myr. The age difference between chromosome X and the autosomes in humans today is an order of magnitude smaller (Supplementary Note 7), again indicating an unusual history in the ancestral population at the time of speciation.

We note that the reduced divergence of humans and chimpanzees on chromosome X also resolves a controversy about mutation rates in males versus females. Comparisons of genomes have shown a lower rate of sequence divergence on chromosome X than the autosomes for many species. On the basis of the hypothesis that this reflects a lower mutation rate in the female germline, it has been used to estimate the ratio α of male:female mutation rates. Studies of human genome repeats¹¹ and human–rat comparisons¹⁴ have indicated that $\alpha \approx 1.9\text{--}2.1$, but comparisons of human and chimpanzee^{12,15} have indicated that $\alpha \approx 6\text{--}7$. The discrepancy can be resolved if the low human–chimpanzee divergence on chromosome

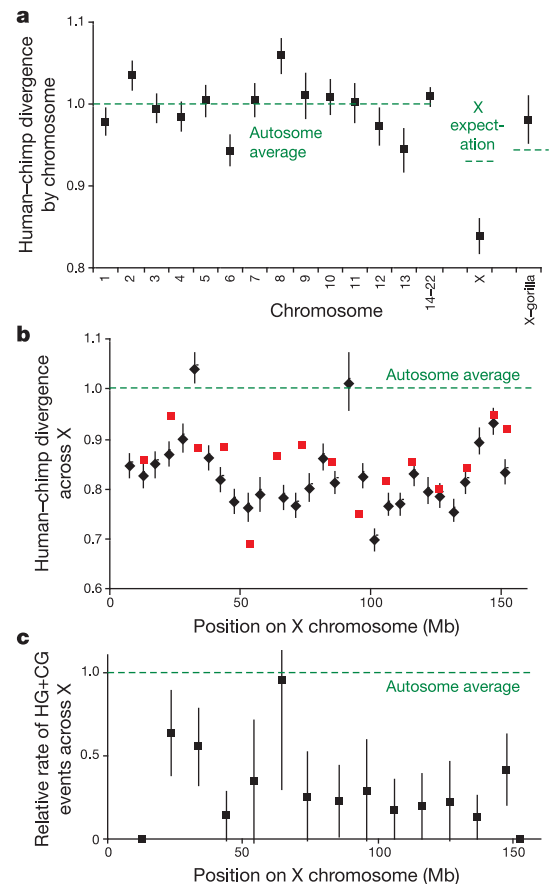


Figure 3 | Reduced human–chimpanzee time divergence across chromosome X. **a**, Human–chimpanzee genetic divergence is lower on chromosome X than on every other chromosome (HCGOM shotgun data, correcting for recurrent mutation), and lower than the theoretical expectation of about 93% for a constant-sized, freely mixing ancestral population (Supplementary Note 5). By contrast, the gorilla chromosome X comparison shows no decrease beyond the expectation of about 95% from theory. We have included a category pooling all chromosomes of less than 100 Mb in size, because the smaller chromosomes do not have much data and thus have large standard errors. **b**, The low time divergence is seen along nearly the entire X chromosome. Black symbols show the ratio of human–chimpanzee to human–macaque divergence plotted in non-overlapping 5-Mb bins in the 15.1 Mb of HCOM alignment. For comparison we also show the ratio of human–chimpanzee to human–gorilla divergence (10-Mb bins) in 372 kb of HCGOM shotgun data (red, ± 1 standard error bars removed for clarity). **c**, The rate of HG and CG sites (HCGOM shotgun data, 10-Mb bins) is also greatly reduced along chromosome X, which is consistent with humans and chimpanzees being most closely related essentially everywhere along chromosome X.

X reflects a low time divergence. Correcting for this, we estimate that $\alpha \approx 1.9$ (Supplementary Note 8), giving no evidence for an increase in α on the primate lineage¹⁶.

Implications for current models of human–chimp speciation

The inference that human–chimpanzee genetic divergence varies over more than 4 Myr, and that genetic divergence is about 1.2 Myr less on chromosome X than the autosomes, raises two issues about human–chimpanzee speciation.

First, these results place an upper bound on the age of human–chimpanzee speciation that poses conflict with some inferences from the fossil record. The Toumaï fossil (*Sahelanthropus tchadensis*), with its bipedalism and hominin dental features, is usually interpreted as being on the hominin line and setting a minimum date for human–chimpanzee speciation^{17,18}. The fossil was originally dated to 6–7 Myr ago (refs 17, 18), and a more recent study estimates 6.5–7.4 Myr ago (refs 19). We compared inferences for the human–chimpanzee speciation date based on the Toumaï fossil with those that would be obtained by extrapolating from older species divergences. We first used an extreme upper bound of 20 Myr ago for human–orangutan genetic divergence²⁰ (Supplementary Table 7). On the basis of the relative genetic divergence of human and chimpanzee (Supplementary Tables 7 and 8; Supplementary Note 9), we infer $\tau_{\text{genome}} < 7.6$ Myr ago and, from the bound $\tau_{\text{species}} < 0.835\tau_{\text{genome}}$, we can infer that $\tau_{\text{species}} < 6.3$ Myr ago. Using a more realistic estimate of human–orangutan genome divergence of less than 17 Myr ago, we obtain a younger bound of $\tau_{\text{species}} < 5.4$ Myr ago. The first bound is not compatible with the older range for Toumaï. The second bound is difficult to reconcile not only with interpretations of Toumaï but also with other fossils recognized as early hominins: *Orrorin tugenensis* at about 5.8 Myr ago (ref. 21) and *Ardipithecus kadabba* at about 5.6–5.8 Myr ago (ref. 22). We emphasize that these calibrations to the older fossil record are not likely to be compromised by molecular clock errors: a ‘rate test’ shows evidence of only slight lineage-specific changes in the mutation rate since the divergence of the great apes²³ (Supplementary Tables 8 and 9). Similar bounds on human–chimpanzee speciation time are obtained by calibration to macaque fossil divergence (Supplementary Note 9).

Second, the properties of chromosome X indicate an unusual evolutionary history around the time of human–chimpanzee ancestral speciation—proving that the structure of the population around the time of speciation was unlike that in any modern human or apes. In a freely mixing population under neutral drift, the ratio R of the genetic divergence on chromosome X and the autosomes should be about 0.75 (the effective population size of chromosome X relative to the autosomes). Such values are, in fact, observed in humans ($R \approx 0.59$ – 0.87) and chimpanzees ($R \approx 0.56$ – 0.76) and inferred for the population ancestral to human and gorilla ($R \approx 0.68$ – 1.00) and chimpanzee and bonobo ($R \approx 0.75$) (Supplementary Note 10). By contrast, the inferred value of R in the population ancestral to human and chimpanzee is 0–0.29 (Supplementary Table 10). We were not able to devise a demographic history consistent with such a low R , even with models of asymmetry between the sexes (Supplementary Note 11). However, strong selection across chromosome X could produce this effect.

The apparent conflict with interpretations of the fossil record could be explained if Toumaï were somewhat younger than previously reported¹⁹, or if there was a problem with the molecular clock used for the calibrations to older fossil divergences. These factors, however, would not explain the more than 4 Myr spread of genetic divergence times across the genome, or the evidence for intense natural selection on chromosome X. We note that, on general grounds, we might expect to see greater evidence for natural selection on chromosome X than the autosomes, because recessive genetic variants are subject to selection in hemizygous males. However, we see no evidence for an unusual X chromosome divergence in the human–gorilla comparison.

Possible hybridization in the human–chimp lineage

We suggest a provocative explanation for multiple features of these data: that the hominin and chimpanzee lineages initially separated but then exchanged genes before finally separating less than 6.3 Myr ago (Fig. 1e). First, this could explain how Toumaï could have dates older than hominin speciation and yet still have hominin features^{17–19}. Second, it could explain the wide range of divergence times (more than 4 Myr): at some loci human and chimpanzee lineages share ancestry around initial separation, whereas at others the genetic ancestry is more recent at the time of hybridization. Third, it could explain the low divergence of human and chimpanzee on chromosome X. An empirically observed pattern, documented in *Drosophila*, tsetse flies, mosquitoes, butterflies and guinea-pigs²⁴, is that “the genes having the greatest effect on hybrid sterility and inviability are X-linked”²⁴. The reasons for this ‘second rule of speciation’²⁴ are not fully understood^{25–27}, although they are thought to be related to Haldane’s rule about hybrid sterility affecting the heterogametic sex more than the homogametic sex²⁸. A corollary—not previously suggested—is that if gene flow between two diverged populations occurs, chromosome X should be subject to strong and rapid selection to eliminate alleles, from one parental population or the other, that contribute to reduced fitness. The presence of multiple hybrid incompatibility loci could lead to selection across much or all of chromosome X, as in our data (Fig. 3). As a specific example, if human and chimpanzee ancestors initially speciated and then interbred, hybrid males might have been infertile, consistent with Haldane’s rule. A viable population could then only have arisen if the fertile females mated back to one of the ancestral populations (for example, chimpanzee ancestors), producing fertile male hybrids when they transmitted X chromosomes derived almost entirely from that ancestral population. This could explain why humans and chimpanzees are most closely related throughout chromosome X. We note that in wild mice in the European *Mus musculus/domesticus* hybrid zone there has been reported to be a gradient of genetic variants on the autosomes, but a sharp geographic transition for chromosome X (ref. 29). This indicates that wild mouse hybrid populations might have difficulty carrying X chromosomes from multiple ancestral populations, which is consistent with what would be expected from our model and proposed corollary to the second rule of speciation.

Speciation in animals is generally believed to occur by allopatry—that is, by the formation of an isolation barrier with no subsequent gene flow. When subsequent hybridization does occur, it is generally believed that the resulting population dies out^{30,31}. However, there are known examples of adapted hybrid populations in nature^{32–34}, and hybridization could be advantageous, allowing nascent species to derive traits from several ancestral populations, combining them to adapt to new environments³⁵. The failure to observe more instances of successful hybridizations in field studies so far^{30,31} might simply be due to ascertainment bias—the fact that hybridizations occur too episodically to be observed practically. With comparative genomic methods, one can project backwards in time to make inferences about what happened at the time at which speciation occurred. Allopatric speciation without subsequent gene flow predicts that population genetic structure before and after speciation should be similar^{30,31}. By contrast, hybridization predicts a wide range of divergence times and different coalescence times in parts of the genome, such as chromosome X. By comparing the genomes of modern species, one could systematically test whether hybridization is a widespread process in evolution.

We have shown that human and chimpanzee speciation was complex; furthermore, our model makes predictions that can be tested with larger data sets³⁶. First, it predicts that evidence of natural selection should be seen not only on chromosome X but also at some autosomal loci. Such ancient selective sweeps might be detected as long regions with unusually low (or high) rates of human–chimpanzee divergence and HG and CG sites. (It might even be

possible to identify autosomal genes under selection). Second, if a hybridization involving a single episode of gene flow occurred, it might result in a bimodal distribution of $\tau(x)$. Third, speciation involving hybridization could give rise to distinctive patterns of 'frozen linkage disequilibrium': differences in the lengths and distribution of HC, HG or CG clustering (Fig. 2). All these hypotheses can be tested once the gorilla genome is complete and aligned to the genomes of humans, chimpanzees and more distant primates.

METHODS

DNA sequence data. We sequenced 115,152 fragments of DNA ('shotgun reads') from a western lowland gorilla and 2,710 from a black-handed spider monkey (*Ateles geoffroyi*) (these data are publicly available in the NCBI trace archive, <http://www.ncbi.nlm.nih.gov/Traces>). We combined our data with whole-genome shotgun sequence data from orangutan and macaque from the Washington University Genome Sequencing Center, the Baylor College of Medicine and the Venter Institute (<http://www.ncbi.nlm.nih.gov/Traces/trace.cgi>; we thank our colleagues for making these data publicly available) (Supplementary Table 1). For a supporting data set, we analysed finished contiguous sequence from bacterial artificial chromosome (BAC) sequencing of sections of chromosomes 7 and X, generated by the NIH Intramural Sequencing Center¹³.

Genome alignments. All shotgun reads were aligned to the NCBI Build 34 human genome assembly using the Arachne³⁷ or BLASTZ³⁸ programs (Supplementary Table 1). At loci with at least 100 base pairs of DNA aligned across all species of interest, we used the Multiple Alignment Program³⁹ to obtain optimized local alignments (Supplementary Methods). We then applied four filters (Supplementary Table 2 and Supplementary Methods) to eliminate the following: first, alignments with an extremely high rate of intraspecific polymorphism; second, alignments with an extreme rate of reads from any one species; third, alignments with a very high rate of divergent sites from some species; or fourth, alignments that mapped to known segmental duplications in humans or chimpanzees⁴⁰. Application of these filters minimized misalignment in our data but did not qualitatively change our main inferences (Supplementary Table 3). The Threaded Block Set Aligner⁴¹ was used for alignment of the BAC data. Aligned data sets are available online and at our website (<http://genepath.med.harvard.edu/~reich>).

Identification of divergent sites for analysis. For the shotgun data, we used divergent sites only if they had a sequencing quality score of at least 30 and 5 bases on each side with quality 25 or more. We additionally required sites to be single nucleotide substitutions, to show exactly two alternate alleles across the species, to be outside hypermutable CpG dinucleotides, to be at least three base pairs from a repeat identified by Tandem Repeat Finder⁴², and to be flanked on either side by at least one base of perfect alignment across species. When multiple reads were available, we used data from the read with the highest sequence quality. Application of these filters did not qualitatively affect the main conclusions from our analysis (Supplementary Table 3). The filtered data sets are available online and at our laboratory website (genepath.med.harvard.edu/~reich).

Genetic divergence estimates. The main measurement in this paper is genetic divergence between two species. To calculate this over a stretch of sequence, we always counted the number of differences per base pair between the two species and normalized by the difference between human and macaque (or another outgroup) over the same stretch. This corrects for variability in mutation rate from locus to locus. In particular, it corrects for a high local mutation rate. As an example for the HCGOM data, the normalized divergence is proportional to $\tau(x) = (n_H + n_{HG} + n_C + n_{CG}) / (n_H + n_{HC} + n_{HG} + n_{HCG} + n_M)$. Using human-macaque divergence for normalization is slightly conservative for X-autosome comparisons. Like all species pairs, humans and macaques are slightly less time-diverged on chromosome X; if we could correct for this, human-macaque divergence on chromosome X could be up to a few per cent less than shown in Supplementary Table 6.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions The authors all played significant roles in the conception, execution, interpretation and presentation of the study.

Author Information To obtain sequencing reads from the NCBI trace archive (<http://www.ncbi.nlm.nih.gov/Traces>), use the following queries:

(1) Gorilla data (*Gorilla gorilla*):

CENTER_NAME = 'WIBR' and CENTER_PROJECT = 'G611'
CENTER_NAME = 'WIBR' and CENTER_PROJECT = 'G612'
CENTER_NAME = 'WIBR' and CENTER_PROJECT = 'G618'
CENTER_NAME = 'WIBR' and CENTER_PROJECT = 'G619'
CENTER_NAME = 'WIBR' and CENTER_PROJECT = 'G744';

(2) New world monkey data (*Ateles geoffroyi*):

CENTER_NAME = 'WIBR' and CENTER_PROJECT = 'G820'.

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Ovulated oocytes in adult mice derive from non-circulating germ cells

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Decades of research in reproductive biology have led to the generally accepted belief that in female mammals, all surviving germ cells enter meiosis at the end of fetal development and as a result, the postnatal ovary harbours a limited supply of oocytes that cannot be replenished or regenerated if lost to injury or disease. However, recent reports have challenged this view, suggesting instead that oocyte production is maintained through continual seeding of the ovary by circulating, bone-marrow-derived germ cells. To test directly the physiological relevance of circulating cells for female fertility, we established transplantation and parabiotic mouse models to assess the capacity of circulating bone marrow cells to generate ovulated oocytes, both in the steady state and after induced damage. Our studies showed no evidence that bone marrow cells, or any other normally circulating cells, contribute to the formation of mature, ovulated oocytes. Instead, cells that travelled to the ovary through the bloodstream exhibited properties characteristic of committed blood leukocytes.

The view that all oocytes capable of participating in female reproduction are present in the ovary at birth has been challenged recently by the surprising observation that the female gonad may exhibit unexpected regenerative activity into adulthood¹. Subsequently, it was suggested that bone marrow and peripheral blood may serve as reservoirs of cells responsible for this regenerative capacity². These reports have led to the proposal that bone marrow or peripheral blood cell transplantation might provide novel treatments for premature menopause or chemotherapy-induced sterility³; however, these findings remain controversial due to residual concerns over the physiological relevance of putative marrow-derived or circulating germ cell precursors and their functional capacity to enter into the pool of mature, ovulated oocytes⁴.

Using acute, intravenous injection of peripheral blood or bone marrow cells from transgenic donor mice, one study² recently reported that oocytes could be restored in the ovaries of chemically or genetically⁵ sterilized female mice within as little as 30 h. It was concluded from these studies that mammalian oocytes are produced postnatally from germline progenitor cells that reside in the bone marrow and travel to the ovaries via the peripheral circulation^{2,3}. To investigate directly the capacity of naturally circulating peripheral blood cells to engraft in the ovary and contribute to oogenesis, we examined ovulated oocytes from adult female mice surgically joined by parabiosis (Fig. 1a). Parabiotic mice develop a common, anastomosed circulatory system within 2–3 days of joining and exhibit continuous, rapid exchange of cells and other circulating factors through the bloodstream^{6,7}. Thus, parabiosis allows direct tracking of genetically marked cells supplied continuously and at physiological levels through the circulation and provides a powerful approach^{7–10} to determine whether blood-borne factors (either humoral or cellular) normally contribute to ovarian function or repair.

Circulating cells do not generate ovulated oocytes

Parabiotic pairs were created by joining wild-type mice with transgenic

animals that ubiquitously expressed green fluorescent protein (GFP) under control of the β -actin promoter^{7,11} (Fig. 1a). GFP-transgenic mice were joined to non-transgenic partners at 4–8 weeks of age, and remained joined for 6–8 months before further analysis. As expected^{7–10}, all parabiotic pairs exhibited high-level peripheral blood chimaerism (on average, ~65% GFP⁺ blood leukocytes in both partners; $n = 4$ pairs, Supplementary Table 1), indicating that a common circulatory system had been established and maintained.

We predicted that if naturally circulating germ cells or their precursors were present in the bloodstream and capable of colonizing the ovary, then as we observed in the blood, GFP⁺ cells from the transgenic parabionts would cross into their non-transgenic partners and colonize their unlabelled ovaries with GFP-labelled oocytes. Similarly, unlabelled germ cells from the non-transgenic animal would cross into the transgenic partner, resulting in GFP-negative oocytes in an otherwise green ovary (Fig. 1b). To test this prediction, we superovulated parabiotic pairs that had been joined for 6–8 months, collected metaphase II oocytes from the oviduct of each partner, and analysed them for expression of GFP (Fig. 1a). In contrast to the substantial chimaerism evident in the peripheral blood of long-term parabionts (Supplementary Table 1), we observed no chimaerism of oocytes in these mice (Fig. 1c and Supplementary Table 1). All oocytes (80 out of 80, $n = 4$ mice) collected from GFP-transgenic partners robustly expressed GFP when examined by direct fluorescence microscopy, whereas none of the oocytes (0 of 66, $n = 4$ mice) collected from non-transgenic partners expressed GFP. Although we occasionally observed GFP⁺ cells associated with the ovulated oocytes in the cumulus masses, staining with antibodies specific to the pan-haematopoietic marker CD45 revealed that these were in fact circulating blood cells (Supplementary Fig. 1). Thus, although circulating cells have the capacity to enter the ovary and to associate with ovulated oocytes, these cells maintain haematopoietic fates in this environment and do not contribute to the production of ovulated oocytes during normal homeostasis.

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No oocytes from circulating cells after ovarian damage

Our analysis of 146 ovulated oocytes collected from animals that shared a common blood circulation for most of their reproductive lifespan revealed no evidence of cross-engraftment of cells capable of generating mature oocytes. Nonetheless, it remained possible that damage to the bone marrow, ovary, or germ cells might be required to enable the ovarian engraftment of circulating cells. To test whether such an injury would enhance cross-engraftment of circulating oocyte precursors in parabiotic mice, we pre-treated non-transgenic mice with the ovotoxic cyclophosphamide (Cytoxan, Cy) and

busulfan (Bu)^{2,12–15}, and then joined them 1 day later with untreated GFP-transgenic partners (Fig. 2a). These animals were maintained as parabionts for ~2 weeks ($n = 4$ parabiotic pairs) or 2 months ($n = 2$ pairs), then induced to superovulate, euthanized for oocyte retrieval, and analysed for chimaerism of oocytes and haematopoietic tissues. As a control we also examined oocytes and blood from parabiotic mice that had been joined for the same length of time, but in which neither partner had been pre-treated by chemotherapy ($n = 2–4$ pairs).

In treated and untreated parabiotic pairs, we observed extensive leukocyte chimaerism in the peripheral blood of both partners (Supplementary Table 2). We also detected significant chimaerism among total bone marrow cells and within the rare subset of blood-forming haematopoietic stem cells (HSCs) in both treated and untreated parabionts. Consistent with the notion that chemoablative damage by Cy/Bu treatment can facilitate engraftment by circulating stem cells, we observed an increase in the level of cross-engraftment of GFP-expressing HSCs into the bone marrow of Cy/Bu-treated non-transgenic mice relative to that observed in the bone marrow of untreated parabiotic controls (Supplementary Table 2). However, in stark contrast to the cross-engraftment evident in the haematopoietic lineages, we found no evidence for cross-engraftment of oocytes in these same animals (Fig. 2 and Supplementary Table 2). Every oocyte (281 out of 281, $n = 12$ mice) collected from GFP-transgenic partners robustly expressed GFP when examined by epifluorescence microscopy. Similarly, none of the oocytes collected from untreated non-transgenic partners (0 out of 154, $n = 6$ mice) or Cy/Bu-treated non-transgenic partners expressed GFP (0 out of 80, $n = 4$, for mice treated 2 weeks before analysis, and 0 out of 4, $n = 2$, for mice treated 2 months before analysis) (Fig. 2b, d and Supplementary Table 2).

In total, we analysed 665 mature, ovulated oocytes from chemotherapy treated and untreated parabiotic pairs and found that they always (665 of 665) possessed the phenotype of the animal from which they were retrieved (Supplementary Tables 1 and 2). In both injured and uninjured non-transgenic parabiotic partners, all GFP⁺ cells associated with the ovulated cumulus complexes expressed CD45 (Supplementary Figs 1 and 3), indicating commitment to the blood cell lineage. Taken together, these data argue against the hypothesis that cells from the bone marrow seed the ovary through the circulation and contribute to functional adult oogenesis, either in the steady state or in response to overt damage to the ovary. Although cells derived from circulation may be found within the ovaries of parabiotic animals, these engrafted cells exhibit exclusively haematopoietic fates, and probably represent circulating blood cells known to infiltrate all tissues in their role as immune responders.

Chemotherapy does not fully deplete oocyte reserves

Although we observed in parabiosis experiments a significant decline in the total numbers of oocytes ovulated by Cy/Bu-treated animals over time, this chemoablative regimen did not cause complete depletion of oocyte reserves in the treated parabionts (Fig. 2c, e and Supplementary Table 2). In parabiotic mice treated with Cy/Bu 2 weeks before superovulation, the numbers of oocytes ovulated were only slightly decreased compared to untreated controls (on average, 20 ± 6 versus 32 ± 13) (Fig. 2c). Similarly, in animals treated with Cy/Bu 2 months before superovulation, ovulated oocytes were substantially reduced in number compared to untreated controls (on average, 2 ± 1 versus 14 ± 4), but not fully eliminated (Fig. 2e). Notably, we found no difference in the number of oocytes ovulated by Cy/Bu-treated mice joined by parabiosis to untreated partners when compared to the number of oocytes ovulated by control (non-parabiosed) animals also treated with Cy/Bu (Supplementary Fig. 2a). These data indicate that Cy/Bu-induced damage, which results in reduced ovulation, is not rescued by factors present in the circulation and supplied through parabiosis.

Incomplete depletion of oocytes in Cy/Bu-treated animals was also apparent upon histological analysis of ovaries retrieved from

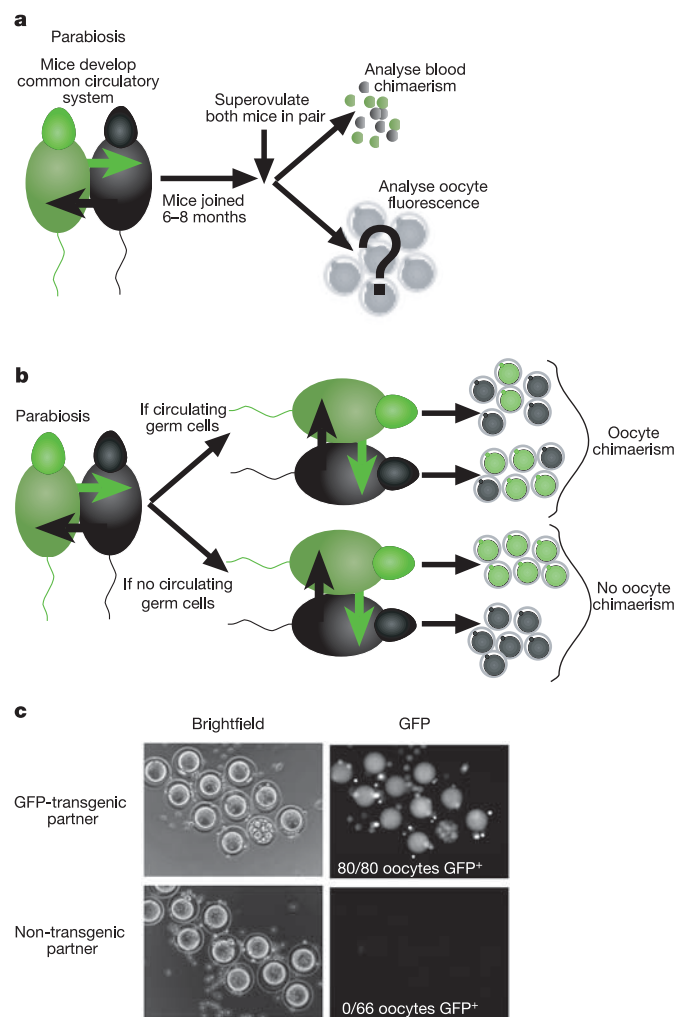


Figure 1 | Circulating cells do not give rise to mature oocytes in long-term parabionts. **a**, Experimental design. GFP-transgenic and non-transgenic C57BL/Ka mice were surgically joined by parabiosis such that they developed a common, anastomosed vasculature. After 6–8 months of shared circulation, pairs were treated with PMS and HCG, and analysed for blood chimaerism and for the presence of cross-engrafting oocytes. **b**, Predictions of the experiments. If circulating germ cells contribute to oocyte formation in adult females, then the population of ovulated oocytes collected from parabiotic mice will contain both host-derived and partner-derived cells (that is, both GFP⁺ and GFP[−] oocytes). If circulating cells do not give rise to oocytes, then all ovulated oocytes collected from parabiotic mice will exhibit the phenotype of the host (that is, GFP⁺ in GFP-transgenic parabionts and GFP[−] in their non-transgenic partners). **c**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected from the GFP-transgenic or non-transgenic partners of pair 9 (Supplementary Table 1). Total numbers of GFP⁺ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 4$ parabiotic pairs). All oocytes from GFP-transgenic parabionts expressed GFP, whereas no oocytes from their non-transgenic partners expressed GFP.

these animals (Supplementary Fig. 2b–g). Ovaries retrieved from untreated control and parabiotic animals contained follicles at many stages of maturation (Supplementary Fig. 2b, f). In contrast, 2 months after treatment with Cy/Bu, the number of follicles was markedly reduced and in some sections, no follicles could be found (Supplementary Fig. 2c, g). However, although Cy/Bu-induced damage to the ovaries was apparently widespread, maturing and mature follicles were observed in some histological sections (Supplementary Fig. 2d, e). The ovaries of both Cy/Bu-treated control and Cy/Bu-treated parabiotic mice showed substantial damage, consistent with the impairment of ovulation observed in these animals (Supplementary Fig. 2c, g).

No ovulated oocytes from transplanted bone marrow cells

Together, these data argue that Cy/Bu-mediated destruction of germ cells is not substantially ameliorated by cells and/or factors naturally

present in the circulation (and therefore provided by parabiosis); however, it remained possible that direct intravenous transplantation of bone marrow cells introduces cells into the circulation that are not normally present in the bloodstream but are capable of contributing to or stimulating oogenesis. To evaluate directly the capacity of transplanted bone marrow cells to contribute to the pool of ovulated oocytes, we examined ovulation efficiency in wild-type mice that had been treated with Cy/Bu and then intravenously infused 24 h later with 4×10^7 unfractionated bone marrow cells. Because our parabiosis experiments indicated that Cy/Bu treatment does not lead to a complete depletion of the oocyte pool, we also examined animals that were sterilized by low-dose total body irradiation (0.5 Gy)—as in the case of ref. 2—and subsequently transplanted with bone marrow cells. Two months after chemotherapy- or radiation-induced damage and bone marrow transplantation, experimental and control animals were induced to superovulate, euthanized for oocyte retrieval, and

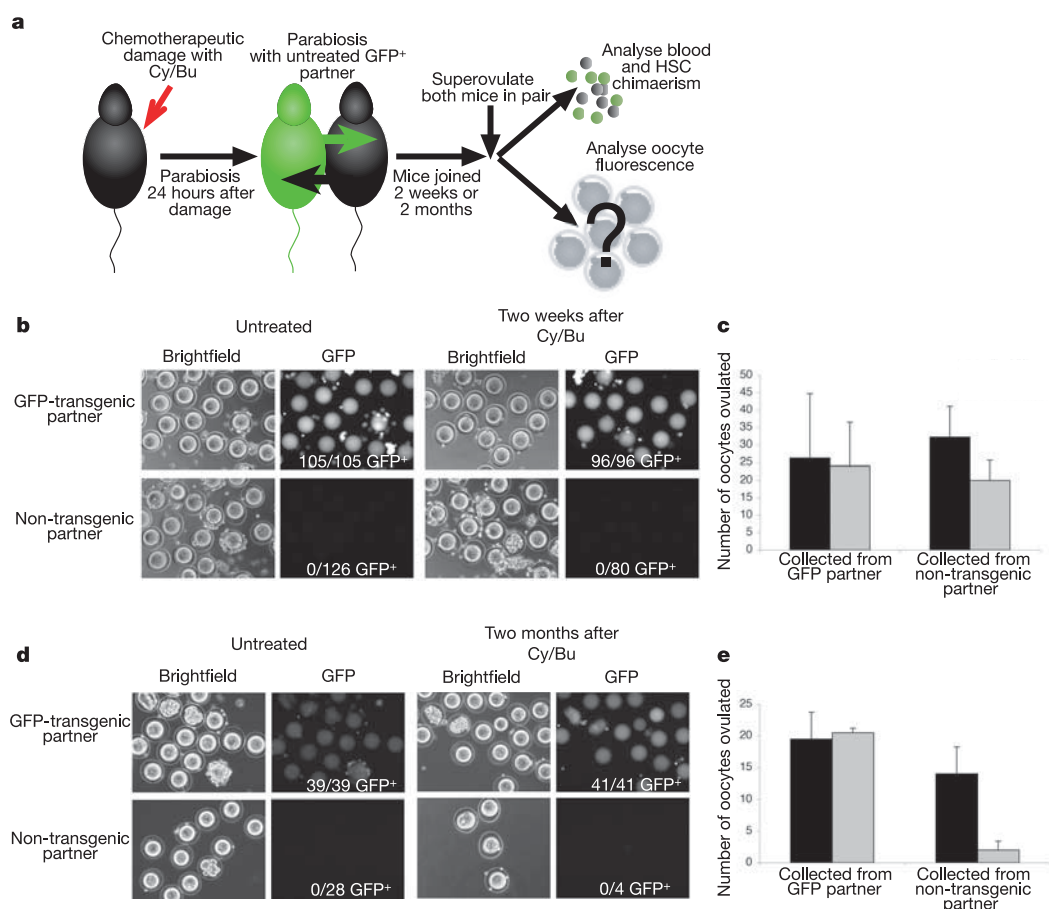


Figure 2 | Circulating cells do not give rise to mature oocytes in injured parabionts. **a**, Experimental design. GFP-transgenic and non-transgenic C57BL/Ka mice were surgically joined by parabiosis 1 day after treatment of the non-transgenic partner only with the ovotoxic agents cyclophosphamide (Cy) and busulfan (Bu). After 10 days or 2 months of shared circulation, pairs were treated with PMS and HCG, and analysed for the presence of cross-engrafting oocytes and/or germ cells. **b**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected after 2 weeks of parabiosis from the GFP-transgenic or non-transgenic partners of untreated pair 5, in which the non-transgenic partner was left untreated, or Cy/Bu-treated pair 8, in which the non-transgenic partner was pre-treated with Cy/Bu (Supplementary Table 2). Total numbers of GFP+ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 4$ parabiotic pairs). **c**, Average number of oocytes ($\pm$ s.d.) collected from parabiotic mice 2 weeks after injury and parabiosis. Black bars represent data from pairs in which neither partner received Cy/Bu treatment; grey bars represent data from pairs in which the non-transgenic

partner only received Cy/Bu treatment. All oocytes from GFP-transgenic parabionts expressed GFP, whereas no oocytes from their non-transgenic partners expressed GFP (see Supplementary Table 2). **d**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected after 2 months of parabiosis from the GFP-transgenic or non-transgenic partners of untreated pair 1, in which the non-transgenic partner was left untreated, or pooled from Cy/Bu-treated pairs 1 and 2, in which the non-transgenic partner was pre-treated with Cy/Bu (Supplementary Table 2). Total numbers of GFP+ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 2$ parabiotic pairs). **e**, Average number of oocytes ($\pm$ s.d.) collected from parabiotic mice 2 months after injury and parabiosis. Black bars represent data from pairs in which neither partner received Cy/Bu treatment; grey bars represent data from pairs in which the non-transgenic partner only received Cy/Bu treatment. All oocytes from GFP-transgenic parabionts expressed GFP, whereas no oocytes from their non-transgenic partners expressed GFP (see Supplementary Table 2).

analysed for chimaerism in haematopoietic tissues and among ovulated oocytes (Fig. 3a).

Low-level haematolymphoid chimaerism was observed in recipient animals receiving GFP-transgenic bone marrow cells and pre-conditioned by either irradiation or cytotoxic drugs (Cy/Bu), but was not observed in untreated recipients (Fig. 3d). These data are consistent with the notion that productive engraftment of transplanted haematopoietic stem cells depends on the immediate availability of 'empty' bone marrow niches, which are rare in untreated animals, but increase in frequency after chemotherapy or irradiation¹⁶. However, in contrast to the haematopoietic engraftment evident in mice transplanted with bone marrow cells expressing GFP, we saw no evidence of GFP⁺ oocytes in these same animals (Fig. 3b, c, $n = 6$).

Equivalent numbers of oocytes were collected from untreated mice (whether untransplanted, transplanted with GFP-transgenic bone marrow, or transplanted with non-transgenic marrow), whereas animals pre-treated with irradiation or cytotoxic drugs showed a marked impairment in their competency to ovulate (Fig. 3e).

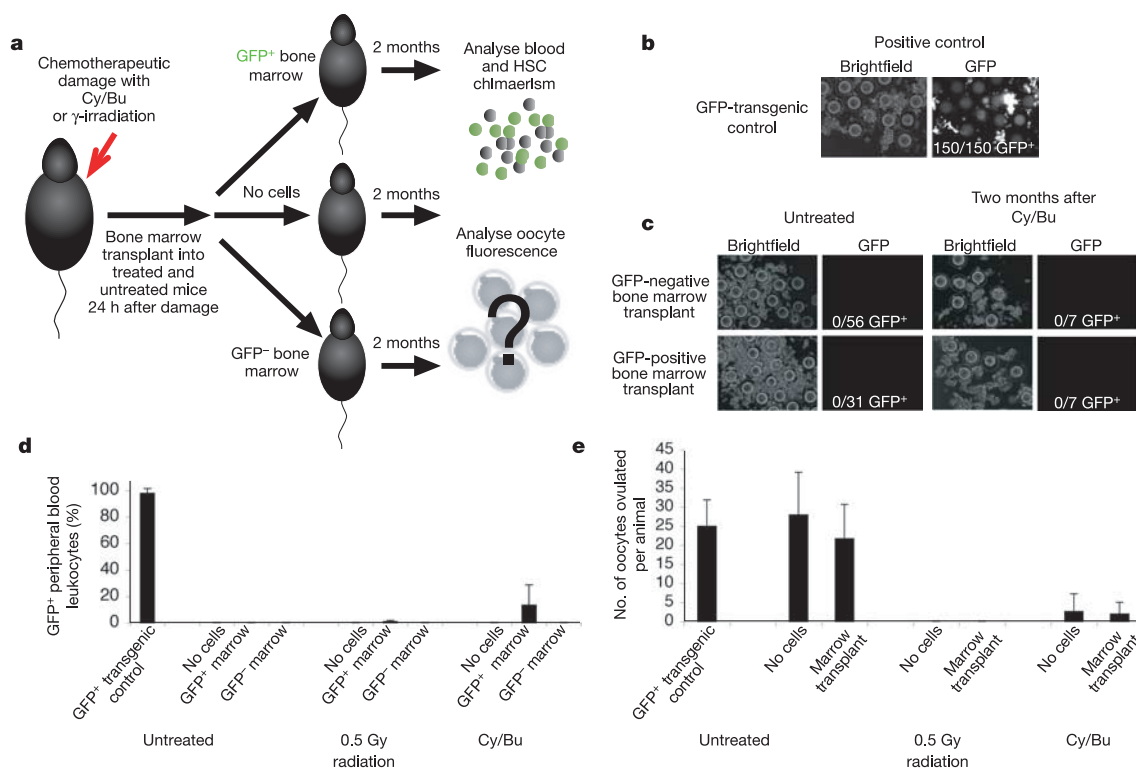


Figure 3 | Bone marrow cells do not give rise to oocytes and do not enhance ovulation of endogenous oocytes in transplanted mice. **a**, Experimental model. Wild-type C57BL/Ka recipients either were pre-conditioned 1 day before transplant by injection of Cy/Bu or by total body irradiation (0.5 Gy), or were left untreated. Mice from each treatment group then received by intravenous injection 4×10^7 GFP-transgenic bone marrow cells or wild-type (GFP⁻) bone marrow cells, or received no cells as a control. Two months later, animals were euthanized for chimaerism analysis of haematopoietic cells and oocytes. **b**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected from GFP-transgenic controls. Total numbers of GFP⁺ oocytes per total oocytes examined are summarized on the epifluorescence panel; all oocytes collected from GFP-transgenic animals were GFP⁺. **c**, Representative brightfield (left) and epifluorescence (right) images of oocytes collected from non-transgenic animals 2 months after transplantation of GFP⁻ or GFP⁺ bone marrow cells. Recipients were untreated before transplant or pre-treated one day previously with Cy/Bu. Total numbers of GFP⁺ oocytes per total oocytes examined are summarized on the epifluorescence panels for each group ($n = 2-6$). No GFP⁺ oocytes were detected in either untreated or Cy/Bu-treated mice receiving GFP⁻ or GFP⁺ bone marrow cells.

Consistent with previous findings², after superovulation no oocytes were found in the oviducts of animals pre-conditioned by 0.5 Gy irradiation, whether or not they received bone marrow transplants. Also consistent with previous results^{2,12-15}, a subset of animals (4 out of 10) pre-treated by Cy/Bu injection produced a small number (on average 2 ± 3) of ovulated oocytes; however, neither the frequency of mice ovulating nor the total number of ovulated oocytes was increased in these animals as a result of bone marrow transplantation (Fig. 3e). Significantly, and as in our parabiosis studies, the ovulated oocytes in Cy/Bu-treated mice (7 out of 7) possessed the phenotype of the animal from which they were retrieved; we found no evidence for the development of GFP⁺ oocytes derived from transplanted cells (Fig. 3c).

Taken together, our data indicate that, unlike low-dose irradiation, Cy/Bu chemotherapy does not completely sterilize female mice and allows a small population of residual endogenous oocytes and/or germ cells to survive and maintain ovulation¹²⁻¹⁵. Importantly, circulating and transplanted bone marrow cells neither contribute directly to the formation of ovulated oocytes in these animals nor do

they enhance endogenous recovery after chemotherapy at the time points examined. Instead, it appears that circulating or bone-marrow-derived cells found in association with ovulated oocytes maintain haematopoietic lineage commitment. It remains a possibility that such circulating or bone-marrow-derived cells that travel to the ovary may in some cases co-express or co-stain with some markers typically associated with female germ cells²; however, the inability of these cells to form ovulated oocytes demonstrates that any such cells that might exist do not contribute to oocyte pools available for fertilization. Finally, the failure of bone marrow transplant and parabiosis to rescue ovulation in Cy/Bu-treated mice indicates to us that bone marrow transplantation is unlikely to reverse or significantly ameliorate premature menopause or chemotherapy-induced sterility. Thus, the findings presented here are consistent with the prevailing theory^{4,17–20} that oocytes in adult mammals are derived from a pool that cannot be regenerated by circulating germ cell progenitors.

METHODS

Mice and antibodies. C57BL/Ka and C57BL/Ka- β -actin/eGFP mouse strains were bred and maintained at Joslin Diabetes Center or at the Harvard School of Public Health. The generation of GFP-transgenic mice has been described elsewhere^{11,21}. GFP-transgenic mice used in these studies were backcrossed for at least 15 generations to C57BL/Ka mice. For parabiosis, animals were joined at 4–8 weeks of age. The monoclonal antibodies used in these studies included OX-7 (anti-Thy1.1, phycoerythrin (PE) conjugate, BD Pharmingen), 2B8 (anti-c-kit, PE-Cy7 conjugate, Ebioscience) and E13-161.7 (anti-Sca-1, Ly6A/E, allophycocyanin conjugate, Ebioscience). The cocktail of haematopoietic lineage (Lin) marker antibodies included KT31.1 (anti-CD3), GK1.5 (anti-CD4), 53-7.3 (anti-CD5), 53-6.7 (anti-CD8), Ter119 (anti-erythrocyte-specific antigen), 6B2 (anti-B220), 8C5 (anti-Gr-1) and M1/70 (anti-Mac-1).

Parabiosis. Parabiosis surgery was performed exactly as described previously^{7,10}, and in accordance with the guidelines established by the Joslin Diabetes Center IACUC for the humane care and use of animals. Where indicated, C57BL/Ka partners were pre-treated by intraperitoneal injection of cyclophosphamide (120 mg kg⁻¹, Sigma) and busulfan (12 mg kg⁻¹, Sigma), as in ref. 2. Treated mice were parabiosed to GFP-transgenic partners 1 day after Cy/Bu treatment.

Bone marrow transplantation. C57BL/Ka recipients were pre-conditioned as in ref. 2, 1 day before transplantation, by intraperitoneal injection of cyclophosphamide (120 mg kg⁻¹) and busulfan (12 mg kg⁻¹) or by 0.5 Gy total body irradiation (delivered from a J.L. Shepard 6810 sealed source ¹³⁷Cs irradiator). Bone marrow cells for transplantation were flushed from the bones of either non-transgenic C57BL/Ka or GFP-transgenic C57BL/Ka- β -actin/eGFP donors into cold Hank's Buffered Saline Solution, containing 2% FBS, using a 21-gauge needle and 1-ml syringe. Cy/Bu-treated, irradiated, or untreated recipients were left untransplanted, or were transplanted intravenously, by retro-orbital injection, with 4×10^7 GFP⁺ or GFP⁻ bone marrow cells. Recipient animals were euthanized 2 months after transplantation for analysis of oocyte and haematopoietic cell chimaerism.

Collection of oocytes. Mice were injected intraperitoneally with 5 international units (IU) of pregnant mares serum (PMS) (Calbiochem) and 48 h later with 5 IU of human chorionic gonadotropin (HCG) (Calbiochem). At 14–15 h after HCG administration, mice were euthanized and oviducts removed. Ovulated oocytes were released from the ampullary region of the oviduct into M2 medium (Speciality Media) supplemented with 0.1% bovine testicular hyaluronidase (Sigma). After dissociation of the cumulus cell–oocyte complexes in hyaluronidase, oocytes were collected into KSOM media (Speciality Media) for epifluorescence analysis and somatic cells from the complexes were subjected to flow cytometric analysis.

Flow cytometry. Haematopoietic chimaerism of parabiotic pairs was determined by flow cytometric analysis of peripheral blood and bone marrow cells after ammonium-chloride-mediated lysis of red blood cells. Chimaerism of oocyte-associated cells and debris collected from the oviduct was also assayed by flow cytometry, without ammonium chloride lysis. For analysis of GFP expression by peripheral blood cells, and of cells from the oviduct, total leukocytes were gated by forward and side scatter characteristics, and dead cells were excluded by staining with propidium iodide. To visualize HSCs, nucleated cells were isolated from whole bone marrow after ammonium chloride lysis of red blood cells and stained first with biotinylated lineage cocktail (Lin, described above), followed by PE–Texas red-conjugated streptavidin (Caltag), PE-conjugated OX-7, allophycocyanin-conjugated E13-161.7, and PE–Cy7-conjugated 2B8. Data were collected on a Coulter Epics cytometer (Beckman

Coulter) maintained at the Diabetes and Endocrinology Research Center (DERC) Flow Cytometry Core Facility at Joslin Diabetes Center or on a FACSaria cytometer (Becton Dickinson) maintained at the Harvard Stem Cell Institute (HSCI) Flow Cytometry Core Facility at the Joslin Diabetes Center. Data were analysed using FlowJo software (Tree Star), and are presented as contour plots of relative fluorescence intensity or tabulated as either the percentage of GFP⁺ leukocytes among total live cells for blood or the percentage of GFP⁺ HSCs among total c-kit⁺Thy1.1^{lo}Lin⁻Sca-1⁺ HSCs.

Epifluorescence analysis. Epifluorescence analysis was performed on ovulated oocytes using an inverted Olympus IX-51 with epifluorescence powered by a super high-pressure mercury lamp. Sequential brightfield and fluorescence images (using a FITC filter) of oocytes were acquired at $\times 20$ magnification using an Olympus U-CMAD3 CCD camera (Olympus) and PictureFrame v2.1 software. Images were saved as high-resolution TIFF files and imported into Adobe Photoshop or InDesign for display purposes.

Histological analysis. Ovaries were fixed in 4% paraformaldehyde at room temperature for 1 h, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Quantification and statistics. Oocytes were counted by visual inspection under brightfield and epifluorescence illumination. Data were analysed for statistical significance using Student's *t*-test (Microsoft Excel).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.J.W., K.E. and R.G. designed the experiments. A.J.W.

performed parabiosis surgeries and transplantations, collected tissues, and analysed haematopoietic chimaerism. K.E. collected oocytes and analysed oocyte chimaerism. I.M.M. and S.J. assisted with flow cytometry and tissue analysis. A.J.W. and K.E. wrote the paper. All authors discussed the results and commented on the manuscript.

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Soft equations of state for neutron-star matter ruled out by EXO 0748–676

F. Özel¹

The interiors of neutron stars contain matter at very high densities, in a state that differs greatly from those found in the early Universe or achieved in terrestrial experiments¹. Matter in these conditions can only be probed through astrophysical observations that measure the mass and radius of neutron stars with sufficient precision². Here I report a determination of the mass and radius of the neutron star EXO 0748–676 that appears to rule out all the soft equations of state of neutron-star matter. If this object is typical, then condensates² and unconfined quarks¹ do not exist in the centres of neutron stars.

The neutron star source EXO 0748–676 has repeatedly shown thermonuclear X-ray bursts. It has also shown, first³ with EXOSAT and very recently⁴ with RXTE, a special type of thermonuclear X-ray burst that is strong enough to lift up the outer layers of the star. During these so-called photospheric radius expansion⁵ bursts, the radiation flux that emerges from the stellar surface is limited by the Eddington flux. It was, however, the detection⁶ of redshifted O and Fe lines by XMM-Newton from the surface of the neutron star that makes this low-mass X-ray binary unique in its class: here multiple phenomena have been observed from a single source that can be concurrently used to determine uniquely the equation of state of its dense interior.

The dependence of the three observable quantities—the Eddington limit, the redshift and the ratio F_{cool}/T_c^4 , which is closely related to the total emitting area—on the stellar properties is shown in Table 1 (F_{cool} is the thermal flux, and T_c is the colour temperature). Even though each of these measurements involve a different combination of the neutron-star mass, radius and distance, when all three observables are known, the expressions can be combined and inverted to yield a unique solution for the three stellar parameters, as shown in Table 2. This is also shown visually in Fig. 1, where the different constraints on the neutron star mass and radius imposed by each observable intersect at a unique set of values. One additional observable that, in principle, can tighten these constraints is the amount of rotational broadening in the redshifted lines. This leads to a direct measurement of the radius of the star⁷, with an uncertainty arising from the angle i that the observer makes with the rotational axis of the star (Fig. 1). For slowly spinning stars, however, other line broadening mechanisms, such as fine structure and Zeeman splitting, may dominate over the rotational broadening^{10,20}. Given the slow spin frequency of EXO 0748–676, rotational broadening alone cannot lead to a meaningful constraint on the stellar radius²⁰.

The only model parameters that are required in this solution are the colour-correction factor f_{∞} and the electron scattering opacity κ_{es} . The colour correction factor f_{∞} , for an observer at infinity, relates the colour temperature to the effective temperature, T_{eff} , of the star by $f_{\infty} \equiv T_c/T_{\text{eff}}$ and can be obtained by theoretical modelling of bursting neutron-star spectra. Here I use a fitting function that describes to an accuracy of 4% the results of recent model atmosphere

calculations⁸ for solar-abundance composition:

$$f_{\infty} = 1.34 + 0.25 \left(\frac{1+X}{1.7} \right)^{2.2} \left(\frac{T_{\text{eff}}^4 (\text{in } 10^7 \text{ K})}{g (\text{in } 10^{13} \text{ cm s}^{-2})} \right)^{2.2} \quad (1)$$

Here, g is the gravitational acceleration at the neutron-star surface. Note that f_{∞} in Tables 1 and 2 corresponds to the colour-correction factor at the cooling tails of the X-ray bursts when the emerging flux is substantially sub-Eddington. At this limit, models of bursting atmospheres⁸ show that f_{∞} asymptotes to a value of ~ 1.34 , practically independent of composition, temperature and stellar gravity, significantly reducing the model dependence of my results. For the electron-scattering opacity I use $\kappa_{\text{es}} (\text{in cm}^2 \text{ g}^{-1}) = 0.2(1+X)$, where $0 \leq X \leq 1$ is the hydrogen mass fraction. This is appropriate for the fully ionized neutron-star atmosphere during the radius-expansion episode. In this calculation, I allow for the entire range of values for X .

The main systematic uncertainty in my results arises from the assumption that the thermonuclear flash engulfs the entire star during the radius expansion and cooling phases of the bursts. There are a number of theoretical and observational arguments that support this assumption. First, numerical models⁹ of the spreading of thermonuclear bursts on the surface of rotating neutron stars show deflagration times that are $\ll 1$ s, which is shorter by orders of magnitude than the duration of the bursts. Second, the constraints¹⁰ on the magnetic field strength of EXO 0748–676, imposed by the lack of Zeeman splitting of the atomic lines, show

Table 1 | The three main quantities observed from EXO 0748–676 and their theoretical dependence on the neutron star properties

Observable	Measurement	Dependence on neutron-star properties
F_{Edd}	$(2.25 \pm 0.23) \times 10^{-8} \text{ erg cm}^{-2} \text{ s}^{-1}$	$\frac{1}{4\pi D^2} \frac{4\pi G M c}{\kappa_{\text{es}}} \left(1 - \frac{2GM}{Rc^2}\right)^{1/2}$
z	0.35	$\left(1 - \frac{2GM}{Rc^2}\right)^{-1/2} - 1$
$F_{\text{cool}}/\sigma T_c^4$	$1.14 \pm 0.10 (\text{km kpc}^{-1})^2$	$f_{\infty}^4 \frac{R^2}{D^2} \left(1 - \frac{2GM}{Rc^2}\right)^{-1}$

The Eddington limit F_{Edd} , defined as the radiation flux at which the outward radiation force balances the inward gravitational force, is the limiting flux emerging from thermonuclear X-ray bursts with photospheric radius expansion. The measurements of the touchdown flux reported here were obtained by averaging the values determined recently with RXTE⁴ and earlier with EXOSAT³ observations, which are consistent with one another. The redshift z of O and Fe absorption lines in the X-ray burst spectra of EXO 0748–676 has been measured for the first time with XMM-Newton⁶. The ratio $F_{\text{cool}}/\sigma T_c^4$, where F_{cool} and T_c are the thermal flux and the colour temperature inferred from the X-ray burst spectra, respectively, asymptotes to a constant value during the cooling tails of the bursts. This ratio is directly related to the emitting surface area of the neutron star and was inferred from the RXTE data⁴ by taking the averages of $F_{\text{cool}}/\sigma T_c^4$ measured 7–15 s after the burst, ensuring that a constant ratio has been reached after the photospheric radius expansion but that the observed flux is still high enough for an accurate determination. The physical constants G and c are the gravitational constant and the speed of light, respectively, $\kappa_{\text{es}} = 0.2(1+X) \text{ cm}^2 \text{ g}^{-1}$ is the electron scattering opacity, X is the hydrogen mass fraction of the accreted material, f_{∞} is the colour-correction factor, and D is the distance to the source. All the above expressions include minimal general relativistic corrections that are accurate in the slow rotation limit, which is appropriate for EXO 0748–676 given its 47-Hz spin frequency¹⁶.

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Table 2 | The neutron star properties that are obtained from the observations summarized in Table 1

Neutron-star property	Dependence on observables	Constraint
M	$\frac{f_{\infty}^4 c^5}{4\sigma_{\text{res}}} \left(\frac{F_{\text{cool}}}{\sigma T_c^4} \right) \frac{(1-(1+z)^{-2})^2}{(1+z)^2} F_{\text{Edd}}^{-1}$	$2.10 \pm 0.28 M_{\odot}$
R	$\frac{f_{\infty}^4 c^3}{2\sigma_{\text{res}}} \left(\frac{F_{\text{cool}}}{\sigma T_c^4} \right) \frac{1-(1+z)^{-2}}{(1+z)^2} F_{\text{Edd}}^{-1}$	$13.8 \pm 1.8 \text{ km}$
D	$\frac{f_{\infty}^2 c^3}{2\sigma_{\text{res}}} \left(\frac{F_{\text{cool}}}{\sigma T_c^4} \right)^{1/2} \frac{1-(1+z)^{-2}}{(1+z)^2} F_{\text{Edd}}^{-1}$	$9.2 \pm 1.0 \text{ kpc}$

The neutron-star mass, radius and distance are uniquely determined from the Eddington luminosity, the redshift, and the ratio $F_{\text{cool}}/\sigma T_c^4$ as shown in the middle column, given a model for the neutron-star atmosphere that determines the colour-correction factor and a measurement of the hydrogen mass fraction. The last column shows the minimum values for the mass, radius and distance to the neutron star for any possible value of the colour-correction factor and the hydrogen mass fraction. The counter-intuitive dependence of the stellar properties on the observables arises from the combination of the expressions shown in Table 1. These expressions also show why a unique spectroscopic determination of the mass and radius of a neutron star, which is necessary to constrain its equation of state, can only be achieved when all three phenomena are observed from a single source, if the distance to the source is unknown.

that the magnetic field is dynamically unimportant and thus cannot inhibit the spreading of the thermonuclear flash over the entire surface.

On the observational side, further evidence for uniform emission from the entire surface as well as the constancy of the Eddington flux during the bursts is obtained from the study¹¹ of the peak luminosity of a large number (~ 70) of bursts from 4U1728–34, which shows that the peak flux is constant to within a few per cent in bursts separated by months. Indeed, a larger study¹² of peak fluxes of all thermonuclear burst sources in the RXTE catalogue also show a quantitatively similar result. Second, in the cooling tails of thermonuclear bursts, the observed ratio F_{cool}/T_c^4 asymptotes to a constant and reproducible value between bursts, strongly indicating that the entire surface of the neutron star emits uniformly in the cooling tails^{13,14}.

Even though flux oscillations of amplitude $\leq 10\%$ have been seen

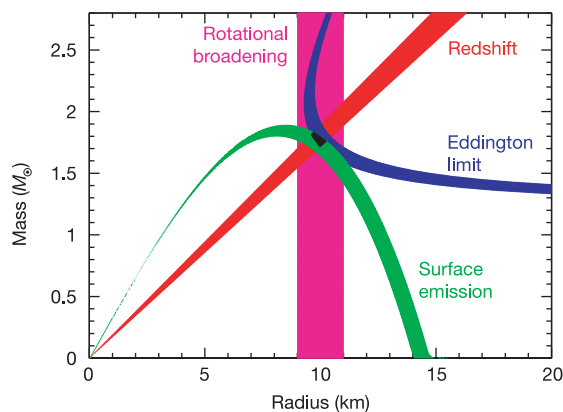


Figure 1 | Four complementary methods to determine the mass and radius of a neutron star. Shown are the contours on the mass-radius plane of neutron stars imposed by the measurement of the Eddington flux during photospheric radius expansion bursts (blue), the ratio F_{cool}/T_c^4 of the surface emission that asymptotes to a constant during the cooling tail of a thermonuclear burst (green), the redshift of atomic absorption lines observed during the burst (red), and the broadening of such lines due to the rotation of the star (magenta) for a hypothetical star with $M = 1.8 M_{\odot}$ and $R = 10 \text{ km}$. The second quantity, obtained from the thermal flux F_{cool} and the colour temperature T_c of the burst spectrum, is closely related to the total emitting area from the stellar surface when the nuclear burst has engulfed the entire star. The widths of the contours correspond to a hypothetical 10% uncertainty in each measurement. The uncertainty in the redshift measurement can be negligible if a grating spectrometer is used, as in the case of EXO 0748–676. The black-shaded area is the intersection of the four contours and corresponds to the uncertainty in the measurement of the true mass and radius of the neutron star.

in some thermonuclear bursts and are thought to be caused by modes excited on the neutron-star surface during the bursts¹⁵, their presence does not increase the uncertainties reported here. Burst oscillations have never been observed during the radius expansion phases of the bursts but only in the rise phase and cooling tails, thus not affecting the determination of the Eddington limit from observations. In addition, the low amplitudes of the oscillations during the cooling tails introduce uncertainties that are smaller than both the systematic and statistical uncertainties (10%) allowed for in my calculations. This is especially true for the burst oscillations in EXO 0748–676, which are very weak, with an average amplitude of 3% (ref. 16).

A final consideration about the Eddington limit is related to the point during a radius expansion burst at which this quantity is measured. The relevant measurement here is the so-called touchdown flux, which is the Eddington limit at the point in the burst when the photosphere returns to the actual radius of the star¹⁴. The flux at the touchdown point cannot be smaller than the Eddington flux because, if the radiation support of the atmosphere were suddenly removed, the photospheric radius would return to the actual size of the star within a free-fall timescale, which is $\sim 1 \text{ ms}$ for a neutron star. Instead, an adiabatic return to the stellar surface, at timescales of a few seconds, is observed in every radius expansion burst, indicating that the emerging flux traces the Eddington limit all the way to the touchdown point.

The only unknown property of the binary system that affects the mass and radius measurements is the hydrogen fraction X of the accreting material. Further observations of the elemental abundances of the accretion flow at long wavelengths could greatly reduce this uncertainty. However, even when I consider the most extreme value of $X = 0.7$, I can obtain lower limits on the mass and radius as follows:

$$M \geq 2.10 \pm 0.28 M_{\odot} \quad \text{and} \quad R \geq 13.8 \pm 1.8 \text{ km} \quad (2)$$

which is shown in Fig. 2. For smaller values of the hydrogen mass fraction, that is, for a helium-rich companion which can be expected

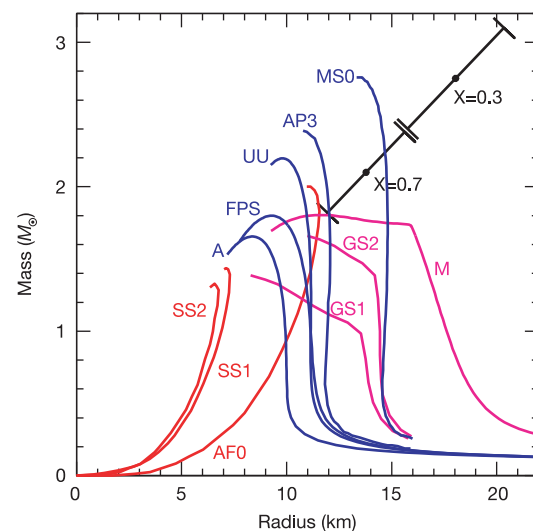


Figure 2 | The constraints on neutron star equations of state imposed by observations of EXO 0748–676. The predicted mass-radius relations for a number of representative equations of state of neutron stars without condensates (blue), with condensates (magenta), and for strange stars (red)^{17–19}. The curve labels and the corresponding references can be found in refs 17 and 18. The two 1- σ error bars correspond to the measurement of the mass and radius of EXO 0748–676 from three independent observations (equation (2)) for different values of the hydrogen mass fraction X of the accreted material. The measurement for $X = 0.7$ corresponds to the minimum allowed values for the mass and radius of the neutron star. Only the stiffest equations of state are consistent with this measurement.

in a small-orbit binary such as EXO 0748–676, the values of M and R are even higher. It is clear from Fig. 2 that the unknown value of X is by far the dominant systematic uncertainty in my result. Also note that even though separate uncertainties on mass and radius are quoted, these are not independent and do not form an error ellipse because the mass-to-radius ratio is fixed by the redshift measurement with a negligible error.

Because I treat distance as an independent variable, I can use the same three measurements to determine a lower limit on the distance to the source, $D \geq (9.2 \pm 1.0)$ kpc. This is a significant advantage of my method: The equations in Table 2 show that if the redshift is measured, the mass and radius measurements are independent of the distance and vice versa. Alternatively, if there is a direct measurement of the distance, for example, for a source in a globular cluster, then only two spectroscopic measurements are sufficient to determine the mass and radius of the neutron star.

The mass–radius measurement presented here does not suffer from uncertainties related to magnetic and centrifugal support that can complicate the calculation of neutron-star structure given an equation of state because of the slow rotation and the low magnetic field of EXO 0748–676. The large size and the high mass of this neutron star impose stringent constraints on the equation of state of matter at supernuclear densities (see Fig. 2). Even the lowest allowed value for the mass and radius can be obtained by only the stiffest equations of state. In particular, equations of state with condensate interiors predict large-radius, low-mass neutron stars (Fig. 2) that are mostly excluded by my measurements. On the other extreme of the mass–radius diagram, self-bound bare strange-matter stars have mostly small masses and radii, also inconsistent with the mass and radius of EXO 0748–676. The more conventional equations of state with neutron–proton compositions are more likely to explain the set of observational properties of EXO 0748–676 presented here. I therefore argue that hadrons and not deconfined quarks represent the ground state of matter.

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LETTERS

Berezinskii–Kosterlitz–Thouless crossover in a trapped atomic gas

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Any state of matter is classified according to its order, and the type of order that a physical system can possess is profoundly affected by its dimensionality. Conventional long-range order, as in a ferromagnet or a crystal, is common in three-dimensional systems at low temperature. However, in two-dimensional systems with a continuous symmetry, true long-range order is destroyed by thermal fluctuations at any finite temperature^{1,2}. Consequently, for the case of identical bosons, a uniform two-dimensional fluid cannot undergo Bose–Einstein condensation, in contrast to the three-dimensional case. However, the two-dimensional system can form a ‘quasi-condensate’ and become superfluid below a finite critical temperature. The Berezinskii–Kosterlitz–Thouless (BKT) theory^{3,4} associates this phase transition with the emergence of a topological order, resulting from the pairing of vortices with opposite circulation. Above the critical temperature, proliferation of unbound vortices is expected. Here we report the observation of a BKT-type crossover in a trapped quantum degenerate gas of rubidium atoms. Using a matter wave heterodyning technique, we observe both the long-wavelength fluctuations of the quasi-condensate phase and the free vortices. At low temperatures, the gas is quasi-coherent on the length scale set by the system size. As the temperature is increased, the loss of long-range coherence coincides with the onset of proliferation of free vortices. Our results provide direct experimental evidence for the microscopic mechanism underlying the BKT theory, and raise new questions regarding coherence and superfluidity in mesoscopic systems.

The BKT mechanism is very different from the usual finite-temperature phase transitions. It does not involve any spontaneous symmetry-breaking and emergence of a spatially uniform order parameter. Instead, the low-temperature phase is associated with a quasi-long-range order, with the correlations of the order parameter (for example, the macroscopic wavefunction of a Bose fluid) decaying algebraically in space. Above the critical temperature this quasi-long-range order is no longer maintained, and the correlations decay exponentially. This picture is applicable to a wide variety of two-dimensional (2D) phenomena, including superfluidity in liquid helium films⁵, the superconducting transition in arrays of Josephson junctions⁶, and the collision physics of 2D atomic hydrogen⁷. These experiments have provided evidence for the BKT phase transition by looking at the macroscopic properties of the system, but could not reveal its microscopic origin—the binding and unbinding of vortex–antivortex pairs^{3,4}.

Harmonically trapped atomic gases generally provide an excellent testing ground for the theories of many-body physics. In particular, they are well suited for the preparation of low-dimensional systems and the detection of individual vortices. Quasi-2D quantum degenerate Bose gases have been produced in single ‘pancake’ traps or at the nodes of one-dimensional (1D) optical lattice potentials^{8–15}. Recently, matter wave interference between small disk-shaped

quasi-condensates has revealed the occasional presence of free vortices¹⁶, but a systematic temperature study was not possible. Theoretically, because the density of states in a 2D harmonic trap allows for finite temperature Bose–Einstein condensation in an ideal gas¹⁷, the nature of the superfluid transition in an interacting gas has been a topic of some debate^{18–24}. Our results indicate that the BKT picture is applicable to these systems, even though in our finite-size system the transition occurs as a finite-width crossover rather than a sharp phase transition²⁵.

We start our experiments with a quantum degenerate three-dimensional (3D) cloud of ⁸⁷Rb atoms, produced by radio-frequency evaporation in a cylindrically symmetric magnetic trap. Next, a 1D optical lattice with a period of $d = 3 \mu\text{m}$ along the vertical direction z is used to split the 3D gas into two independent clouds and to

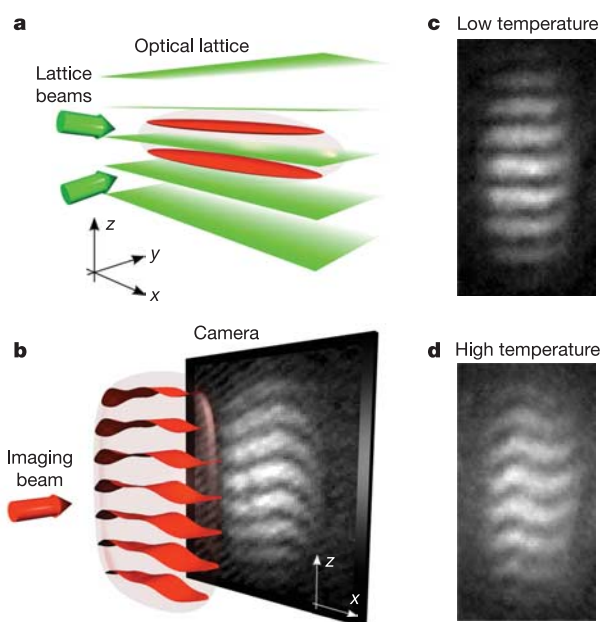


Figure 1 | Probing the coherence of 2D atomic gases using matter wave heterodyning. **a**, An optical lattice potential of period $d = 3 \mu\text{m}$ along the vertical direction z is formed by two laser beams with a wavelength of 532 nm intersecting at a small angle. It is used to split a quantum degenerate 3D gas into two independent planar systems. The transparent ellipsoid indicates the shape of the gas before the lattice is ramped up. **b**, After the confining potential is abruptly switched off, the two atomic clouds expand, overlap and interfere. The interference pattern is recorded onto a CCD camera using the absorption of a resonant probe laser. The waviness of the interference fringes contains information about the phase patterns in the two planar systems. **c**, **d**, Examples of interference patterns obtained at a low and a high temperature, respectively.

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compress them into the 2D regime (Fig. 1a). To minimize heating and ensure thermal equilibrium, the lattice potential is ramped up slowly over 500 ms, and the clouds are allowed to equilibrate for another 200 ms. At full laser power, the height of the lattice potential is $V_0/h = 50$ kHz, where h is Planck's constant. At this lattice height, the tunnelling between the two planes is negligible on the timescale of the experiment, and the motion along the tight confining direction z is 'frozen out'^{14,16}. The two clouds form parallel, elongated 2D strips, characterized by the harmonic trapping frequencies of 11 Hz, 130 Hz and 3.6 kHz along the x , y and z directions, respectively. The number of condensed atoms per plane is a function of temperature and varies between 0 and 5×10^4 , whereas the total atom number per plane is $\sim 10^5$. For the largest (quasi-)condensates, the Thomas–Fermi approximation yields $120 \mu\text{m}$ and $10 \mu\text{m}$ for the x and y lengths of the strips, respectively. The corresponding chemical potential and healing length are respectively $\mu/h = 1.7$ kHz and $\xi = 0.2 \mu\text{m}$.

After the trapped 2D gases have equilibrated, all confining potentials are abruptly turned off. The two clouds expand predominantly perpendicular to the x – y plane and, as they overlap, a 3D matter wave interference pattern forms²⁶. After $t = 20$ ms of 'time-of-flight' (TOF) expansion, the projection of the 3D interference pattern onto the x – z plane is recorded on a CCD camera, using a resonant probe laser directed along y (Fig. 1b). At any fixed position x , the interference pattern along z is characterized by its contrast $c(x)$ and phase $\varphi(x)$. To extract these two parameters, we fit the density distribution with a function:

$$F(x, z) = G(x, z)[1 + c(x) \cos(2\pi z/D + \varphi(x))]$$

where $G(x, z)$ is a gaussian envelope, $D = h/mv$ is the period of the interference fringes, and m is the atomic mass. The function $c(x)$ is a measure of the local coherence in the 2D clouds (with some coarse grain averaging due to the integration along the imaging axis y), while the variation of $\varphi(x)$ with x is a measure of the long-range coherence. With increasing temperature, the presence of phase fluctuations in the two planes increases the waviness of the interference fringes, that is, the fluctuations in $\varphi(x)$ (compare Fig. 1c and d).

In order to explore different temperature regimes for the 2D gas, we vary the final radio frequency ν_{rf} used in the evaporative cooling of the initial 3D gas. The temperature $T^{3\text{D}}$ is proportional to $\Delta\nu = \nu_{\text{rf}} - \nu_{\text{rf}}^{(\text{min})}$, where $\nu_{\text{rf}}^{(\text{min})}$ is the final radio frequency that completely empties the trap. We explore the range between the onset of condensation in the 3D gas ($T^{3\text{D}} = 150$ nK) and a quasi-pure 3D Bose–Einstein condensate. As the lattice is ramped up, the temperature of the compressed gas can increase significantly (2–3 times), but precise direct thermometry in the lattice is difficult. Instead, in order to quantify the degeneracy of the 2D system, we measure the local contrast in the centre of the interference pattern, $c_0 = \langle c(0) \rangle$, where $\langle \dots \rangle$ denotes an average over many images recorded under the same experimental conditions (temperature and atom number).

The dependence of c_0 on the initial $T^{3\text{D}}$ (that is, $\Delta\nu$) is shown in Fig. 2. The interference fringes are visible for $\Delta\nu < 35$ kHz, which closely corresponds to the range of condensation in the initial 3D gas. As $\Delta\nu$ is lowered, c_0 grows smoothly. For $\Delta\nu$ below ~ 12 kHz, the initial 3D Bose–Einstein condensate is essentially pure and c_0 saturates at about 30%. In an ideal experiment, the expected contrast at zero temperature is $c_0 = 1$. The finite resolution of our imaging system limits the maximal observable contrast to about 60%. We attribute the difference between expected and measured maximal contrasts to the residual heating of the gas in the optical lattice, caused in particular by the three-body recombination processes. This hypothesis is supported by the fact that the atoms experience the lattice potential over 700 ms, which is not negligible compared to the measured lifetime of 2.5 s for the atom cloud in the lattice. In the following, we use c_0 rather than $T^{3\text{D}}$ as a direct measure of the degeneracy of the 2D gas.

We now turn to a quantitative analysis of long-range correlations

as a function of temperature. The coherence in the system is encoded in the first-order correlation function:

$$g_1(\mathbf{r}, \mathbf{r}') = \langle \psi^*(\mathbf{r})\psi(\mathbf{r}') \rangle$$

where $\psi(\mathbf{r})$ is the fluctuating bosonic field at position $\mathbf{r}$. From interference signals recorded at different positions along the x axis, one can extract information about g_1 , as well as higher-order correlation functions²⁷. Here we adopt an analysis method proposed in ref. 28. The idea is to partially integrate the 3D interference pattern over lengths L_x and L_y , along the x and y directions respectively, and study how the resulting contrast C decays with the integration lengths. Specifically, in a uniform system and for $L_x \gg L_y$, the average value of C^2 should behave as:

$$\langle C^2(L_x) \rangle \approx \frac{1}{L_x} \int_0^{L_x} dx [g_1(x, 0)]^2 \propto \left(\frac{1}{L_x} \right)^{2\alpha} \quad (1)$$

The long-range physics is then captured in a single parameter, the exponent α , which describes the decay of $\langle C^2 \rangle$ with L_x . The expected values of α may be understood in two simple limits. In a system with true long-range order, g_1 would be constant and the interference fringes would be perfectly straight. In this case $\alpha = 0$, corresponding to no decay of the contrast upon integration. In the opposite limit, if g_1 decays exponentially on a length scale much shorter than L_x , the integral in equation (1) is independent of L_x . In this case $\alpha = 0.5$, corresponding to adding up local interference fringes with random phases¹⁴. One of the central predictions of the BKT theory is that at the transition, the superfluid density should suddenly jump to a finite value that is a universal function of the transition temperature²⁹. When adapted to the interference measurements with uniform 2D Bose gases²⁸, this 'universal jump in superfluid density' corresponds to a sudden drop in α from 0.5 to 0.25.

In our experiments, integration along y is automatically performed in absorption imaging, with $L_y \approx 10 \mu\text{m}$ fixed by the size of the quasi-condensates. Our system is also not uniform along x , and the average local contrast $c_x = \langle c(x) \rangle$ decreases smoothly towards the edges of the quasi-condensate owing to the increasing effects of thermal excitations. For comparison with theory, we consider the integrated contrast:

$$\tilde{C}(L_x) = \frac{1}{L_x} \left| \int_{-L_x/2}^{L_x/2} c(x) e^{i\varphi(x)} dx \right|$$

This would exactly coincide with C in a uniform system. We extract the exponent α using only the quasi-uniform region where $c_x > 0.5c_0$. Figure 3a shows examples of the measured $\langle \tilde{C}^2 \rangle$ as a

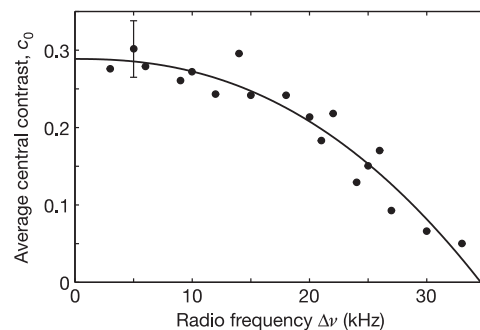


Figure 2 | Local coherence as a thermometer. The average central contrast c_0 of the interference patterns is plotted as a function of the parameter $\Delta\nu$ controlling the temperature of the 3D gas before loading the optical lattice. The solid line is a fit to the data using the empirical function $c_0 = c_{\text{max}}[1 - (\Delta\nu/\Delta\nu_0)^\gamma]$, with $c_{\text{max}} = 0.29 \pm 0.2$, $\Delta\nu_0 = 35 \pm 1$ kHz and $\gamma = 2.3 \pm 0.4$. The total number of images used for the plot is 1,200, corresponding to 41 measurements of c_0 . Different measurements of c_0 taken at equal $\Delta\nu$ have been averaged. The displayed error bar indicates the largest standard deviation.

function of L_x at a low and a high temperature, along with the fits by a power-law decaying function.

Figure 3b summarizes the fitted values of the exponent α in different temperature regimes, and constitutes the first main result of this Letter. Starting at high temperatures, for values of c_0 up to about 13%, α is approximately constant and close to 0.5. When the temperature is reduced further, α rapidly drops to about 0.25, and for even lower temperatures (larger c_0) it levels off. We thus clearly observe a transition between two qualitatively different regimes at high and low temperatures. The values of α above and below the transition are in agreement with the theoretically expected jump in the superfluid density at the BKT transition in a uniform system. However, this quantitative agreement might be partly fortuitous. Even though we concentrated on the quasi-uniform part of the images, the geometrical effects in our elongated samples could still be important. Ultimately, at extremely low temperature, α should slowly tend to zero and the gas should become a pure, fully coherent Bose–Einstein condensate. We could not reach this regime in the present experiments owing to the residual heating discussed above.

Even without precise thermometry, we can estimate the cloud's temperature and density at the onset of quasi-long-range coherence. For images with $c_0 = 0.15$, the temperature inferred from the wings of the atom distribution after TOF is 290 ± 40 nK, corresponding to a thermal wavelength of $\lambda = 0.3 \mu\text{m}$. From the length of the quasi-condensate we deduce the number of condensed atoms $N_C = 11,000 \pm 3,000$, and the peak condensate density (in the trap centre) $\rho_C = (5 \pm 1) \times 10^9 \text{cm}^{-2}$. This gives $\rho_C \lambda^2 = 6 \pm 2$. BKT theory for a uniform system predicts the transition at

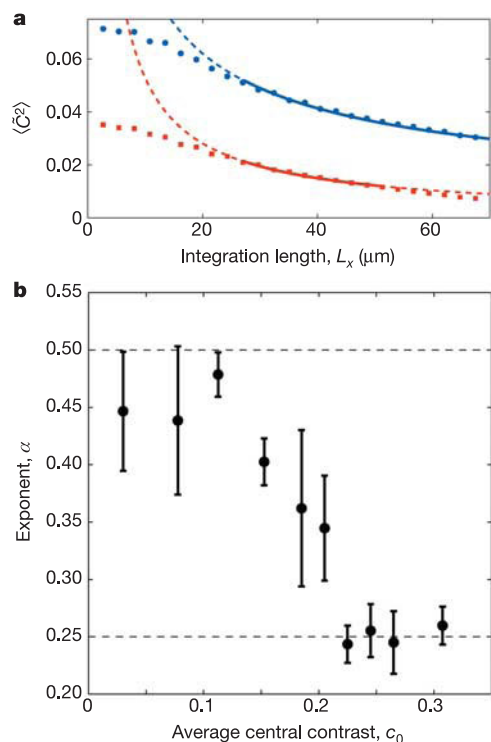


Figure 3 | Emergence of quasi-long-range order in a 2D gas. **a**, Examples of average integrated interference contrasts $\langle \bar{C}^2(L_x) \rangle$ are shown for a low (blue circles, $c_0 = 0.24$) and a high (red squares, $c_0 = 0.13$) temperature; L_x is the integration length. The lines are fits to the data by the power-law function $1/(L_x)^{2\alpha}$, and give $\alpha = 0.29 \pm 0.01$ (low temperature) and $\alpha = 0.46 \pm 0.01$ (high temperature). The fitting range, indicated by the solid part of the line, is constrained by the conditions $L_x \gg L_y$ on the left and $c_x > c_0/2$ on the right. **b**, Decay exponent α as a function of c_0 . Dashed lines indicate the theoretically expected values of α above and below the BKT transition in a uniform system. Error bars indicate the standard deviation of the results from different experimental runs.

$\rho_S \lambda^2 = 4$, where ρ_S is the superfluid density. The two values are in fair agreement, but we note that the exact relation between ρ_C and ρ_S in 2D atomic gases will require further experimental and theoretical investigation. For example, our observation of $\alpha \approx 0.5$ for a finite value of c_0 suggests that the superfluid density ρ_S might be zero even if the condensate density ρ_C is finite.

The key role in the microscopic BKT theory is played by vortices, localized topological defects in the phase of the condensate. In contrast to the smooth variation of the fringe phase $\varphi(x)$ created by long-wavelength phonons (Fig. 1d), a free vortex in one of the condensates should appear as a sharp dislocation in the interference pattern^{16,24}, with $\varphi(x)$ changing abruptly across a dislocation line parallel to the expansion axis z . We indeed occasionally observe such dislocations. Examples of images containing one and several dislocations are shown in Fig. 4a and b, respectively. The tightly bound vortex–antivortex pairs are not detectable in our experiments because they create only infinitesimal phase slips in the interference pattern. Other phase configurations which could mimic the appearance of a vortex, such as a dark soliton aligned with the imaging direction, can be discarded on theoretical grounds²⁴.

Figure 4c shows the frequency with which we detect sharp dislocations at different temperatures. For the count we consider only the central, 30- μm -wide region of each image, which is smaller than the length of our smallest quasi-condensates. We note that we detect only a subset of vortices—those that are well isolated and close to the centre of the cloud. We also note that thermally activated phonon modes with a very short wavelength along x can in principle contribute to the count. Their contribution is expected to be non-negligible only at the highest temperatures, at which a detailed theoretical analysis would be needed to separate their effect from that of the vortices.

The observed sudden onset of vortex proliferation with increasing temperature constitutes the second main result of this Letter. Further, this onset coincides with the loss of quasi-long-range coherence (Fig. 3b). These two observations together provide conclusive evidence for the observation of the BKT crossover in this system.

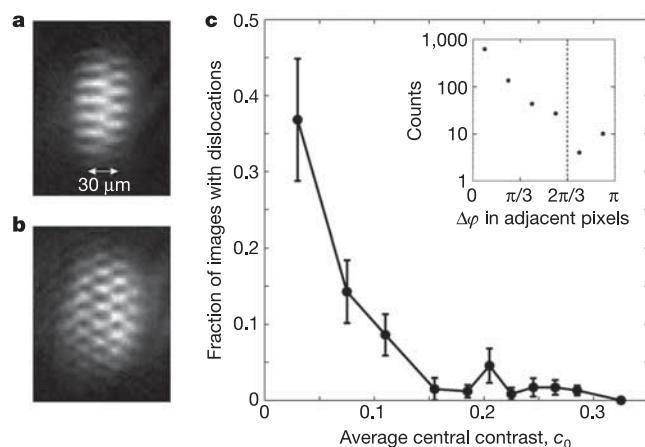


Figure 4 | Proliferation of free vortices at high temperature. **a**, Example of an interference pattern showing a sharp dislocation that we attribute to the presence of a free vortex in one of the interfering clouds. **b**, Interference pattern showing several dislocations. **c**, Fraction of images showing at least one dislocation in the central, 30- μm -wide region, plotted as a function of c_0 . The error bars show the statistical uncertainty, given by the square root of the number of images with dislocations. Inset, histogram of the phase jumps $\Delta\varphi_i = |\varphi(x_i) - \varphi(x_{i+1})|$ between adjacent CCD pixel columns, for the set of images in the bin $c_0 = 0.08$. An image is counted as showing a dislocation if at least one of the $\Delta\varphi_i$ exceeds $2\pi/3$ (threshold indicated by the dashed line). The distance between adjacent columns is $2.7 \mu\text{m}$ and the count runs over the 10 central columns. There are 97 images contributing to this histogram, hence 970 counts, among which 16 counts (corresponding to 13 different images) exceed the threshold.

Our experiments support the notion that the unbinding of vortex–antivortex pairs is the microscopic mechanism destroying the quasi-long-range coherence in 2D systems. The related question of the superfluidity of the sample remains open. It could be addressed in the future by setting the planar gases in rotation and studying the ordering of the vortex lattice. Alternatively, a study of the damping of the collective eigenmodes of the gas could be used to infer its viscosity. Our experiments may also raise new theoretical questions related to the geometry and the mesoscopic nature of the system.

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LETTERS

Mesostructured germanium with cubic pore symmetry

Gerasimos S. Armatas¹ & Mercouri G. Kanatzidis¹

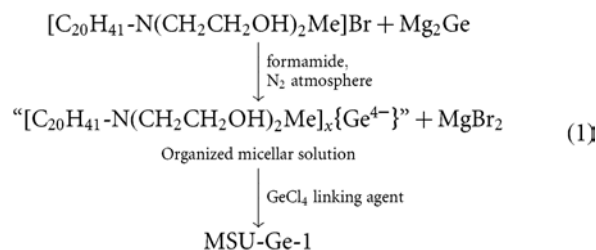
Regular mesoporous oxide materials have been widely studied^{1–8} and have a range of potential applications, such as catalysis, absorption and separation. They are not generally considered for their optical and electronic properties. Elemental semiconductors with nanopores running through them represent a different form of framework material with physical characteristics contrasting with those of the more conventional bulk, thin film and nanocrystalline forms¹. Here we describe cubic mesostructured germanium, MSU-Ge-1, with gyroidal channels containing surfactant molecules, separated by amorphous walls that lie on the gyroid (G) minimal surface as in the mesoporous silica MCM-48 (ref. 2). Although Ge is a high-melting, covalent semiconductor that is difficult to prepare from solution polymerization, we succeeded in assembling a continuous Ge network using a suitable precursor for Ge⁴⁺ atoms. Our results indicate that elemental semiconductors from group 14 of the periodic table can be made to adopt mesostructured forms such as MSU-Ge-1, which features two three-dimensional labyrinthine tunnels obeying *Ia3d* space group symmetry and separated by a continuous germanium minimal surface that is otherwise amorphous. A consequence of this new structure for germanium, which has walls only one nanometre thick, is a wider electronic energy bandgap (1.4 eV versus 0.66 eV) than has crystalline or amorphous Ge. Controlled oxidation of MSU-Ge-1 creates a range of germanium suboxides with continuously varying Ge:O ratio and a smoothly increasing energy gap.

The liquid-crystal templating mechanism for achieving synthetic control on the nanoscale has led to the discovery of inorganic mesophases with remarkable pore architectures. This methodology involves electrostatic interactions and charge matching at the interface of the self-assembled surfactant molecules and inorganic species. Originally used with silica-based materials², this approach was extended to organosilicates^{3,4} and transition metal oxides^{5–8}. Whereas the mesoporous ceramic oxides are desirable for catalytic, separation and adsorption applications, their frameworks lack exciting electronic and optical properties. The idea of semiconducting mesoporous framework solids led various research groups to develop synthetic routes for non-oxidic materials by binding chalcogenide clusters with a variety of linkage metal ions^{9–11}. These materials demonstrate long-range ordering with optoelectronic properties that originate from the surfactant/chalcogenide framework.

In contrast to mesostructured compounds, mesostructured elemental materials have not been developed and only a few examples involving transition-metal systems have been reported, including Pt and Pt/Ru alloys^{12,13}. The synthesis of elemental structures perforated with tunnels on the nanoscale is challenging because of the difficulty in growing or organizing appropriate precursors in a desired manner. Elemental mesoporous semiconductors with a three-dimensional arrangement of pores could modulate their physical parameters in such a way that new opto-electronic properties not present in the bulk analogues can be achieved^{9,14}.

The semiconductor germanium, with its many technological applications, has been studied extensively^{15–17}. A large number of different methods of synthesis and developing of Ge nanoparticles have been reported^{18,19}. Recently, Ge nanocrystals prepared in the liquid phase using a non-anionic surfactant as the size- and shape-directing agent has been described^{20,21}. Although the synthesis of germanium nanocrystals is well established, with procedures that affect the size and shape of the 'bulk' nanoparticles, no work has been reported for the synthesis of Ge semiconductors with mesostructured frameworks. Here we report a successful synthetic strategy for the preparation of a mesostructured Ge semiconductor, based on a liquid-crystal-templated technique and a new form of framework based entirely on Ge with a nanopore system of cubic gyroid symmetry.

To produce the mesostructured germanium, we used Ge⁴⁺ anions derived from Mg₂Ge and linked them via a metathesis reaction with germanium tetrachloride (GeCl₄) in the presence of cationic surfactants; see scheme (1). The synthesis must be carried out under an inert atmosphere. The amphiphilic surfactant *N*-eicosane-*N*-methyl-*N,N*-dis(2-hydroxyethyl)ammonium bromide (EMBHEAB) was used as the micellar template in formamide solution. The prepared hybrid organic–inorganic material labelled MSU-Ge-1 has the formula EMBHEA-[Ge]_m (where *m* ≈ 8):



The powder X-ray diffraction (XRD) pattern of MSU-Ge-1 is shown in Fig. 1a. The pattern exhibits several well-resolved peaks in the low-angle range $2\theta = 2\text{--}8^\circ$, which can be indexed as (211), (220), (321), (400), (420), (332), (422), (431), (611) and (543) diffraction peaks; this pattern corresponds to the cubic *Ia3d* space group with a refined cubic unit-cell parameter of $a = 8.4 \text{ nm}$. The large number of observed Bragg peaks in the XRD pattern indicates a high pore order and long-range periodicity in the structure. Furthermore, the XRD results reveal that the framework of germanium maps onto the gyroidal minimal surface, analogous to that of the mesoporous silicate MCM-48 (Fig. 1b)²².

The chemical composition of MSU-Ge-1 was determined with elemental C,H,N and energy-dispersive microprobe analysis. The analysis confirmed the presence of Ge only and the absence of any Mg or halide element (Cl or Br); see Fig. 1c. The composition of MSU-Ge-1 was consistent with the stoichiometry of EMBHEA-[Ge]₈.

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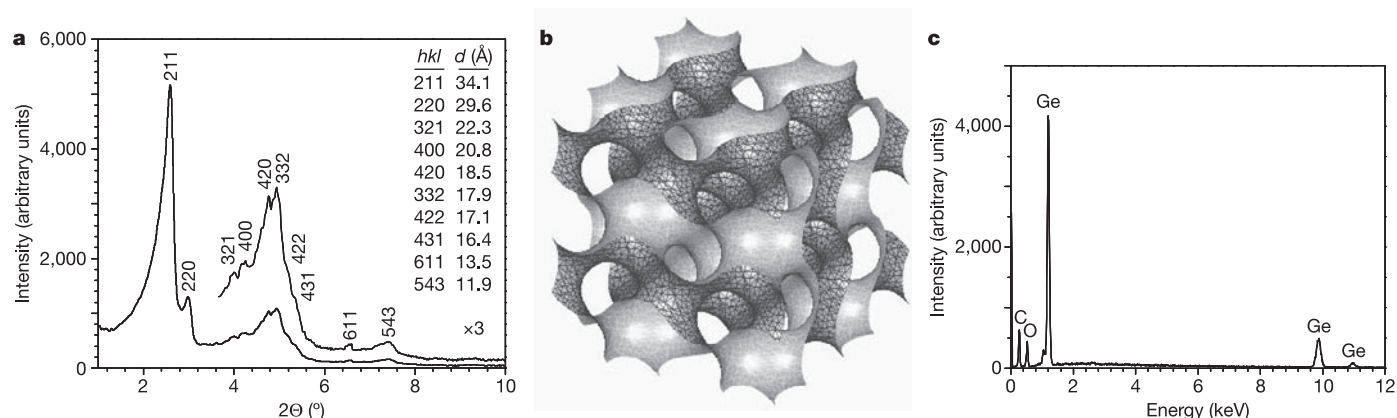


Figure 1 | X-ray scattering of mesostructured MSU-Ge-1, bicontinuous gyroid minimal surface and energy-dispersive X-ray spectrum. a, Powder XRD pattern of cubic mesostructured germanium. This pattern is similar to those reported for high-quality cubic silica MCM-48 systems. The indexing of the Bragg reflections (see inset) is consistent with a body-centred unit cell with $a = 8.4$ nm. **b**, The bi-continuous gyroid minimal surface with $Ia\bar{3}d$

pore symmetry. The elemental germanium framework follows the gyroid surface and the molecules of surfactant occupy the pore channels. The two different shades of surface belong to the two independent channel systems in the structure. **c**, Energy-dispersive X-ray spectrum of purified MSU-Ge-1 showing the presence of only Ge element. The carbon- and oxygen-related peaks are attributed to the surfactant in the material and possibly the grid.

Figure 2 presents typical transmission electron microscopy (TEM) images obtained from MSU-Ge-1, and the corresponding fast Fourier transforms (FFT) taken along a thin area of the images. We succeeded in recording TEM images along the $[100]$, $[110]$, $[111]$ and $[311]$ directions, which revealed regular and extended pore periodicity confirming that the material is a cubic gyroidal mesostructure²¹. Furthermore, indexing of the diffraction spots of FFTs (insets in Fig. 2) confirms that the periodic structure is defined by body-centred-cubic symmetry in the $Ia\bar{3}d$ space group. From the TEM

studies, the size of the surfactant and the XRD-determined pore–pore spacings we estimate a Ge wall thickness of ~ 1 nm.

The absence of Bragg peaks in the higher-angle region ($2\theta > 10^\circ$) of the XRD pattern establish the non-periodic structure of the Ge framework in MSU-Ge-1. However, the local structure of the framework was probed with X-ray diffuse scattering and pair distribution function (PDF) analysis²³. Figure 3a shows the PDF plots as a function of interatomic distance r for MSU-Ge-1 and polycrystalline germanium. The PDF plot exhibits strong interatomic correlation

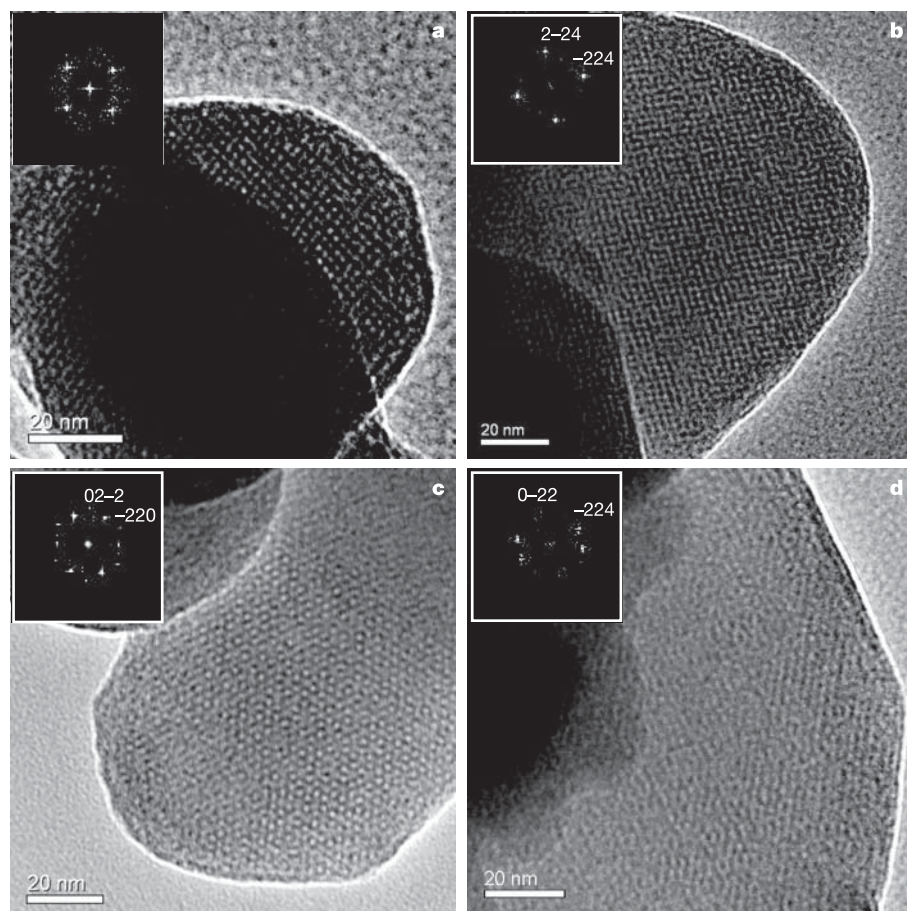


Figure 2 | TEM images of mesoporous germanium semiconductor MSU-Ge-1. a, Image taken along the $[100]$ direction; **b**, along the $[110]$ direction; **c**, along the $[111]$ direction and **d**, along the $[311]$ direction. The pore–pore spacings are consistent with those deduced from XRD. Insets in each panel show FFT images taken along an area of the high-resolution TEM images.

vectors at 2.3 and 4.0 Å, which correspond to Ge–Ge bonds and Ge···Ge next-nearest-neighbour distances in the mesoporous framework. By comparison, polycrystalline Ge (Fig. 3a) and amorphous Ge²⁴ shows the same two correlation peaks at 2.4 and 4.0 Å originating from the Ge–Ge bonds and Ge···Ge second-neighbour distances and indicating local structure similar to that of MSU-Ge-1. This suggests that the MSU-Ge-1 framework has a well-defined local structure (that is, Ge–Ge bonding); however, because of the very thin walls (~1 nm) of the gyroid Ge network we expect most of the Ge atoms to be located on the surface. Therefore, we cannot be sure that the full tetrahedral geometry of crystalline or amorphous Ge is in fact present in MSU-Ge-1. The lack of the atomic pair correlation peaks at distances beyond 5 Å confirms the absence of intermediate or long-range order in the Ge network of MSU-Ge-1. Crystalline Ge exhibits longer-range interatomic correlation peaks owing to its periodicity. Importantly, the PDF lacks correlation peaks at 1.7 and 3.2 Å, which would arise from Ge–O bonds and Ge–O–Ge moieties in the structure²⁵.

Although oxide species in the mesostructured framework of MSU-Ge-1 are not expected on the basis of the anaerobic conditions of scheme (1), their presence was probed with Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy is sensitive to atom–atom vibrations and the characteristic band can be identified even if a small amount of Ge–O bonds existed in the structure. The spectrum of the as-prepared semiconductor indicated a lack of stretching vibrations at 904 and 956 cm^{−1} that is typical of Ge–O bonding. This supports the PDF analysis results discussed above. Evidence for oxide species (GeO_x) on the germanium framework began to appear in samples after extended exposure (over 2 days) to air, judging from the growth of the intense and broad Ge–O band centred at 924 cm^{−1} (see Fig. 3b).

The mesostructured germanium framework of MSU-Ge-1 exhibits optical and electrical properties different from those of the bulk crystalline or amorphous phases. Figure 3c shows the optical absorption spectra of the as-prepared MSU-Ge-1 and crystalline Ge as well as those obtained after extended exposure of MSU-Ge-1 to ambient air (over 10 h and 2 days). There is a clear and substantial blueshift of

the absorption edge in MSU-Ge-1 relative to bulk Ge by ~0.76 eV. That is, the absorption associated with the bandgap transition occurs at 1.42 eV for MSU-Ge-1 versus 0.66 eV for Ge. This marked blueshift can be understood by considering the changes in the density of the electronic energy states due to the thin walls of the Ge network in MSU-Ge-1 (~1 nm). We observed similar blueshifted bandgaps of ~1.4 eV in Ge nanocrystals ~4 nm in diameter—a consequence of quantum size effects²⁶.

The gradual bandgap increase in the oxidation products of MSU-Ge-1 (Fig. 3c) suggests the generation of novel sub-oxide GeO_x species in the framework. The formation of GeO_x causes a successive widening of the energy gap involving the conversion of Ge–Ge bonds to Ge–O–Ge moieties. This was confirmed by PDF of the air-oxidized species, which shows correlation peaks at 1.8, 2.9 and 4.2 Å (see Supplementary Fig. S1). These are interatomic correlations from Ge–O bonds, Ge···Ge second neighbours (via the Ge–O–Ge moieties) and Ge···Ge distant neighbours (see Supplementary Fig. S1). The end stage of this process is presumably GeO₂ which has a bandgap of 4.56 eV. The continuous blueshift with degree of oxidation indicates a homogeneous process and an evolution from Ge to GeO_x, and not a generation of a mixture of MSU-Ge-1 and GeO₂ phases. The controlled air oxidation of MSU-Ge-1 is topotactic (as judged by the XRD patterns of air-oxidized sample which indicate retention of the gyroid and the *Ia3d* space group; see Supplementary Fig. S2) and leads to a mesostructured material. Therefore the range of GeO_x compositions generated in this fashion represents a novel class of materials worthy of future studies of its own.

The thermal stability of MSU-Ge-1 was examined with thermogravimetric (TGA) and differential TGA analysis in a nitrogen atmosphere (Fig. 3d). The TGA profile shows a weight loss (14%) below 250 °C occurring in broad steps due to removal of physisorbed and possibly chemisorbed formamide. This is followed by a gradual weight loss of 42.5% occurring between 250 and 460 °C, which corresponds to the decomposition of surfactants that fill the mesopores. The material obtained at 600 °C was shown to be crystalline Ge.

Mesoporous semiconductors should be a fertile ground for physico-chemical investigations²⁷. Application possibilities may arise now

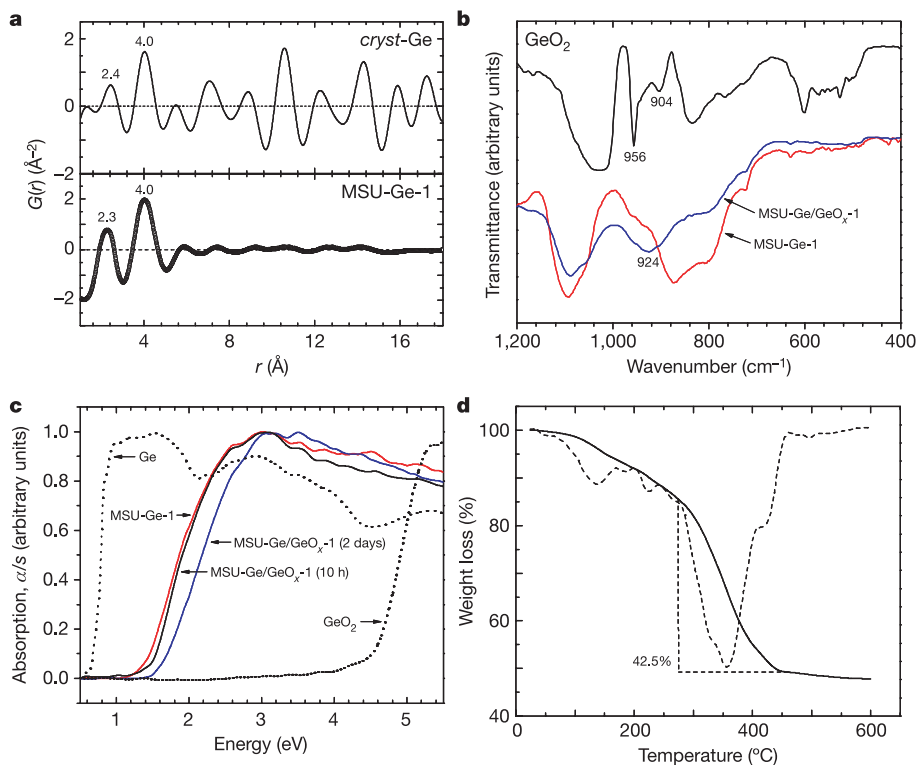


Figure 3 | PDF analysis, FTIR and optical absorption spectra and TGA data. **a**, Reduced atomic PDF $G(r)$ as a function of the interatomic distance r of mesostructured Ge and microcrystalline Ge (*cryst-Ge*) materials. Amorphous Ge shows a similar $G(r)$ function to that of MSU-Ge-1 (ref. 24). **b**, FTIR spectra of MSU-Ge-1 (red line), and the sample after extended exposure in the air (~2 days) (blue line) as well as crystalline GeO₂. The intense peak centred at 924 cm^{−1} of the oxidized MSU-Ge-1 material is attributed to broadening of the Ge–O vibration bands at 904 and 956 cm^{−1} (all spectra were recorded in diffuse reflectance mode). **c**, Optical absorption spectra of MSU-Ge-1 (red line) and oxidized material after 10 h exposure (black line) and after 2 days exposure to air (blue line). The results reveal energy bandgap 1.42, 1.47 and 1.61 eV for the MSU-Ge-1 and oxidized versions, respectively. The absorption spectra of bulk microcrystalline Ge (0.66 eV) and GeO₂ (4.56 eV) are also given for comparison. (α and S are the absorption and scattering coefficients, respectively.) **d**, TGA profile of as-prepared elemental mesoporous germanium (under nitrogen flow of 20 ml min^{−1}). The weight loss 42.5% in the temperature range 250–460 °C, indicated from the differential TGA curve (dashed line), we attribute to the combustion of surfactant.

that we can study and exploit the semiconducting properties of the Ge network, and in a broader sense those of mesoporous materials based on elements. Moreover, the approach of using elemental Zintl anions (as in Mg_2Ge) as a template seems particularly suited to constructing a wide range of elemental porous materials—and could generate a class of mesostructured elements with novel combinations of properties, such as medium or narrow energy gaps, light absorption properties or dopability. We have also found (work to be published elsewhere) that a mesoporous germanium network of the MCM-41 type constructed from Ge-clusters can also be made by a surfactant-templated process. These observations open up the possibility of general synthetic routes to ordered mesoporous elements with a variety of pore architectures, including mesoporous silicon.

METHODS

Preparation of Mg_2Ge precursor and cationic surfactant. The Zintl compound Mg_2Ge was prepared according to a modified literature preparation²⁸, inside a glovebox under nitrogen atmosphere. Ge powder and Mg metal with 10% molar excess were heated at 650 °C for 3 days in a sealed Nd tube, enclosed in an evacuated silica ampoule. The purity of the blue-grey product was confirmed with powder XRD. The amphiphilic surfactant EMBHEAB was prepared by reacting stoichiometric amounts of 1-bromoeicosane with *N*-methyldiethenolamine in ethanol under reflux conditions for 48 h. Pure surfactant compound was obtained by recrystallizing twice from CHCl_3 –ethyl acetate mixture solution²⁹.

Preparation of MSU-Ge-1. A typical procedure for the preparation of MSU-Ge-1 is as follows: finely ground powder of Mg_2Ge (48 mg, 0.04 mmol) was suspended in 5 ml formamide at 75 °C under continuous stirring for 18 h. The mixture was allowed to cool to 65 °C and the surfactant (280 mg, 0.06 mmol) was added. The mixture was stirred for 5 h and heated slowly to 75 °C. A solution of 0.04 mmol GeCl_4 in 1 ml of formamide was then added dropwise to this mixture using a pipette and the resulting mixture was aged overnight at 85 °C (the GeCl_4 solution must be added immediately upon preparation within a period of approximately 2 min). The light brown-grey product was isolated by filtration, washed with warm formamide several times and dried under vacuum at room temperature overnight to yield MSU-Ge-1 in ~83% yield. All manipulations were carried out in a glovebox filled with nitrogen. To remove any dense Ge phase the as-prepared material was suspended in chloroform (1 mg ml^{-1}) while stirring for 20 min. The less-dense MSU-Ge-1 forms a stable suspension whereas the minor byproducts sink. The suspension was decanted carefully with a pipette and the pure product was isolated after filtration, washed with chloroform and dried under vacuum at room temperature for 12 h.

Elemental C,H,N analysis was performed in a Perkin Elmer Series II CHNS/O Analyser 2400 instrument. The sample was heated at 160 °C for 1 h under N_2 flow before the measurements. The results were consistent from sample to sample and the average was C 32.24%, H 5.97%, and N 1.61%.

Attempts to remove the surfactant from mesostructured germanium by thermal decomposition led to contraction of the inorganic framework with consequent loss of pore ordering. For example, heating at 400 °C for 3 days, although it removes most of the surfactant (only 8% remains), leads to a shrinkage of the unit-cell volume by 19% and lowering of the pore periodicity. The specific surface area on such thermally treated material was found to be 18 $\text{m}^2 \text{g}^{-1}$ by nitrogen adsorption measurements. The thermal treatment was as follows: the samples were heated from 30 to 230 °C (5° min^{-1}) for 3 h and then from 230 to 400 °C (5° min^{-1}) for 3 days under a nitrogen flow of 30 ml min^{-1} .

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LETTERS

Hexagonal nanoporous germanium through surfactant-driven self-assembly of Zintl clusters

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Surfactant templating is a method that has successfully been used to produce nanoporous inorganic structures from a wide range of oxide-based material^{1–5}. Co-assembly of inorganic precursor molecules with amphiphilic organic molecules is followed first by inorganic condensation to produce rigid amorphous frameworks and then, by template removal, to produce mesoporous solids. A range of periodic surfactant/semiconductor and surfactant/metal composites have also been produced by similar methods^{6–11}, but for virtually all the non-oxide semiconducting phases, the surfactant unfortunately cannot be removed to generate porous materials. Here we show that it is possible to use surfactant-driven self-organization of soluble Zintl clusters to produce periodic, nanoporous versions of classic semiconductors such as amorphous Ge or Ge/Si alloys. Specifically, we use derivatives of the anionic Ge_9^{4-} cluster^{12–14}, a compound whose use in the synthesis of nanoscale materials is established¹⁵. Moreover, because of the small size, high surface area, and flexible chemistry of these materials, we can tune optical properties in these nanoporous semiconductors through quantum confinement^{16,17}, by adsorption of surface species, or by altering the elemental composition of the inorganic framework. Because the semiconductor surface is exposed and accessible in these materials, they have the potential to interact with a range of species in ways that could eventually lead to new types of sensors or other novel nanostructured devices.

In developing the self-organization chemistry of reactive non-oxide clusters, a number of synthetic challenges must be overcome. First, although water is generally the solvent of choice for promoting amphiphilic assembly, it is far too reactive for these syntheses. Formamide has been used in the past with non-oxide clusters^{9,10}, but even this solvent is too reactive. To promote amphiphilic assembly, a network-forming (hydrogen-bonding) solvent is needed¹⁸—we use ethylenediamine, which uniquely balances the need for donor–acceptor pairs (N: and N–H) to promote assembly with an intolerance of acidic protons. The use of this solvent may open other possibilities for amphiphilic self-organization using reactive precursor molecules.

In the synthesis of these composites, a surfactant/ethylenediamine solution is combined with a similar solution of a polymer version of the Ge_9^{4-} cluster; the overall goal is to produce ordered composites through inorganic/organic co-organization followed by oxidative crosslinking of the clusters and template removal. We chose a surfactant with a large head group—cetyltriethylammonium bromide, CTEAB—to encourage formation of the curved hexagonal phase¹⁹. We use polymer $(\text{Ge}_9^{2-})_n$ rather than the monomeric Ge_9^{4-} because the crosslinked nature of the precursor apparently lowers the entropic penalty associated with self-organization.

Immediately on mixing the surfactant solution and the Zintl cluster solution, a precipitate forms which produces the ‘as-synthesized’

low-angle X-ray diffraction (XRD) pattern shown in Fig. 1a (black line). The peaks can be indexed to a hexagonal honeycomb structure with lattice constant $a = 4.12$ nm and plane group $p6mm$. Low-angle scattering shows well-defined nanometer-scale order, but high-angle XRD (not shown) indicates that these materials are amorphous on the atomic length scale. C, N and Ge elemental analyses (Fig. 1a inset) confirm that the as-synthesized composite is neutral, with the chemical formula $(\text{CTEA})_2\text{Ge}_9$. One additional peak at $\sim 10^\circ$ is also observed in both the as-synthesized composite and a sample of pure $(\text{Ge}_9^{2-})_n$ polymer (Fig. 1a, dotted line). The peak probably originates from small-angle scattering from the individual Zintl clusters that make up these samples. Therefore this material, although it is nanoporous, does not have crosslinked Ge walls, but rather is made up of Ge_9 clusters.

To confirm this hypothesis, we used Ge extended X-ray absorption fine structure (EXAFS) analysis. This technique is a sensitive probe of first- and second-shell bonding and allows us to determine when we lose the Ge_9 clusters. Figure 2a and c shows raw and Fourier-transformed EXAFS data for as-synthesized composites. We fitted the EXAFS using crystallographic data from the $(\text{Ge}_9^{2-})_n$ polymer to generate a model¹⁴, considering the four unique atom sites (Fig. 2c, right, black atoms). The model includes coordination distances only up to 0.51 nm and thus is unable to fit peaks at large radial distance. FEFF 7 was used to generate phase and amplitude factors for each germanium site in a multiple scattering formalism²⁰, and these were combined with appropriate weighting to generate an overall model. The Debye–Waller factor, the relative bond lengths, and the exact edge position were optimized to produce the best fit to the data. The agreement between the data and fit are not perfect, but there is good general agreement between all of the first four peaks of the model and the data. Given that the model is based on crystallographic data and the composites are clearly amorphous, the agreement is quite remarkable. Relatively small Debye–Waller factors of $0.0025\text{--}0.005 \text{ \AA}^2$ also indicate that the Zintl cage is retained in the composite.

Because the Ge walls are not well interconnected, attempts to directly remove surfactant from as-synthesized composites results in loss of nanoscale periodicity. For silica-based systems, robust materials that can be made porous are produced through dehydration of silanol groups to form Si–O–Si linkages¹. For the Ge system, the analogous chemistry is the oxidative coupling of two anionic Ge species to form Ge–Ge bonds. We use mild oxidizing conditions to prevent the formation of GeO_x ; ferrocenium hexafluorophosphate was used to partially oxidize the anionic Ge towards neutral Ge and crosslink the framework. Low-angle XRD (Fig. 1a, dashed line) shows that the peak shifts towards higher q (smaller repeat distance) upon oxidation, indicating a more condensed network. In addition, the Zintl cluster peak at $q = 0.635 \text{ \AA}^{-1}$ is completely removed by this treatment. Some disordering of the nanoscale structure also occurs,

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however, because well-resolved hexagonal higher-order diffraction peaks are no longer observed after oxidation. Despite this, transmission electron microscopy (TEM) images of partly oxidized samples (Fig. 1b and d) show clear nanoscale order with a periodic repeat distance that agrees with low-angle XRD. Because the grains of this nanostructured Ge power are rod-like with the pores running along the length of the rod (Fig. 1e), the pattern most often observed in TEM is stripes. Occasionally, however, a grain can be found standing on end, which produces the hexagonal array of pores observed in Fig. 1d.

The oxidation process serves not only to crosslink the framework and break up the Ge_9 clusters, but also to remove part of the surfactant template. A combination of optical absorption and ^1H nuclear magnetic resonance (NMR) analysis on the reacted ferrocenium solution, and elemental analysis of the partly oxidized composite shows that the ferrocenium is oxidized quantitatively to ferrocene and the surfactant, ion paired with the hexafluorophosphate, is liberated quantitatively into solution (see Fig. 1a inset and Supplementary Fig. S2). Unfortunately, oxidation alone cannot remove all the surfactant because oxidation beyond 70% of the anionic charge results in degradation of the nanoscale structure.

To produce a porous material, the remaining cationic surfactant must be replaced with a smaller cationic species, and the clear choice for this is a proton. In fact, in the absence of hydrogen, pure amorphous Ge is not stable. Theoretical studies have shown that 4-coordinated amorphous networks are overstrained and unstable²¹, and thus bulk amorphous Ge is actually Ge:H . By using H^+ instead of H_2 , we combine surfactant ion-exchange with dangling bond termination. Because of the high reactivity of the Ge framework, we use a supported acid (a proton exchange resin) to provide a proton source in the absence of both water and a reactive conjugate base. Again ^1H NMR and elemental analysis (Fig. 1a inset) indicate that

surfactant is liberated into solution during this proton treatment, although a small amount of organic material remains in the composite ($\leq 10\%$). Brief aqueous HF treatment can further increase the extent of hydrogen passivation.

Low-angle XRD of the proton-treated composite is shown in Fig. 1a (grey and dot-dashed lines). A continued shift to higher q (smaller repeat distance) indicates some additional shrinkage of the composite upon surfactant removal and H-passivation. The final materials show hexagonal lattice constants of 3.3–3.5 nm, although like the sample that was only partially oxidized but not proton treated, clear higher-order hexagonal peaks still cannot be observed. Comparison of the partially oxidized and oxidized/proton-treated samples, however (Fig. 1), indicates that little additional disordering occurs upon proton treatment. TEM images of the final proton-treated material again show nanometre-scale periodicity (Fig. 1c).

EXAFS analysis of data collected on the ferrocenium-oxidized proton-treated sample is shown in Fig. 2b and d. In agreement with XRD data, the EXAFS fits poorly to the cage model used for the as-synthesized composite. Previous work on bulk $\alpha\text{-Ge:H}$ has shown that this tetrahedral solid can be effectively fitted with a first-shell tetrahedral model²², and this model fits our composites extremely well (Debye–Waller factor = $0.006 \text{ \AA}^2$). The presence of a small peak around $R + \delta \approx 4 \text{ \AA}$ may be assigned to second-shell tetrahedral Ge coordination. The results provide strong evidence that the Zintl-based inorganic framework has been converted to simple amorphous Ge. This conclusion is corroborated by Ge X-ray photoelectron spectroscopy (XPS) (Supplementary Fig. S1) which shows only the presence of zero valent Ge. The majority of the Ge is in the form of elemental Ge, with a small amount of GeC , presumably formed from the reaction of decomposing surfactant with the pore wall²³. The presence of some Ge–C bonds is consistent with the small amount of

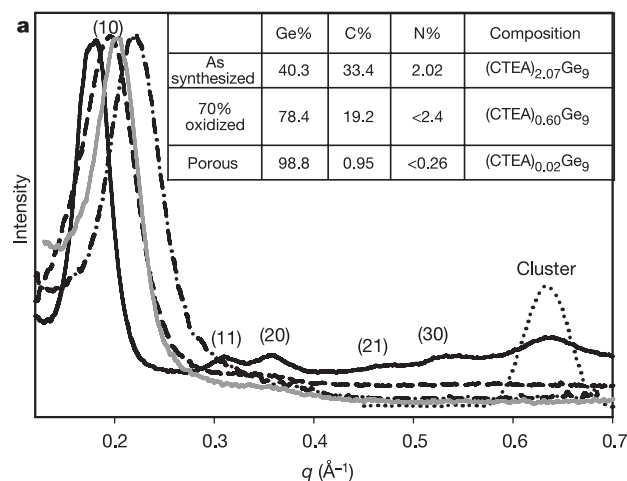
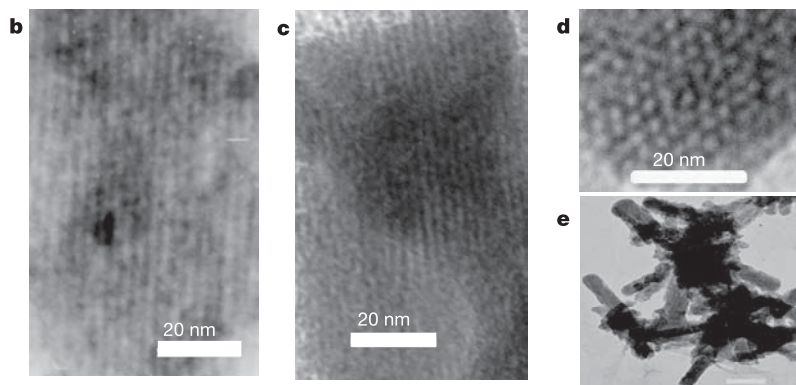


Figure 1 | TEM and X-ray diffraction on hexagonal Ge. **a**, Low-angle X-ray diffractions from germanium surfactant composites after various chemical treatments: as-synthesized composite (black), composite after oxidation (dashed), ion-exchanged porous Ge (grey), porous Ge after HF wash (dot-dashed). The as-synthesized composite shows five peaks in a $1:\sqrt{3}:2:\sqrt{7}:3$ ratio that can be indexed to a $p6mm$ hexagonal phase. When this as-synthesized composite is chemically treated, the intensity of the higher-order peaks is reduced, suggesting that oxidation produces a more condensed but slightly less ordered framework. In the as-synthesized composite, a peak corresponding to the size of the cluster can also be observed at $q = 0.635 \text{ \AA}^{-1}$. This peak can also be observed in a sample of the pure $(\text{Ge}_9^{2-})_n$ polymer (dotted), but not in the oxidized or nanoporous samples. The inset table lists elemental analysis for these samples. **b**, TEM image of a partly oxidized composite; **c**, TEM image of porous germanium; **d**, TEM image showing hexagonal periodicity in a partly oxidized composite; **e**, particle size distribution for the porous Ge by TEM.



residual carbon in the proton-treated sample that cannot be removed by ion exchange or solvent wash. No signal from oxygen or any alkali cations is observed.

The porosity of the treated samples can be analysed using nitrogen porosimetry. The data in Fig. 3a, which shows a type-H4 isotherm²⁴, was collected on a ferrocenium-oxidized (70 mol.%), proton-treated composite that was washed with acetonitrile. The isotherm shows a step at the nitrogen fragility point and so the desorption step cannot be used to determine the pore size distribution. Such an isotherm is usually characteristic of slit-like pores, rather than perfect cylinders²⁵. In our case, this probably results from the small amount of organic material that remains in the pores. Despite this fact, the Brunauer–Emmett–Teller (BET) surface area is measured to be $500 \text{ m}^2 \text{ g}^{-1}$. Normalizing for the relative densities of silica and germanium, this value corresponds to $\sim 1,200 \text{ m}^2 \text{ g}^{-1}$ surface area in a silica-based material, consistent with surface areas reported for ordered silica materials with similar repeat distances¹. The results indicate that the majority of the surfactant can be removed from these composite materials to produce porous, nanostructured germanium. Although a range of well-ordered non-oxide semiconducting surfactant/inorganic composites has been synthesized^{6,8–10}, these Ge-based materials are unique in that the surfactant can be removed without destroying the nanometer-scale structure.

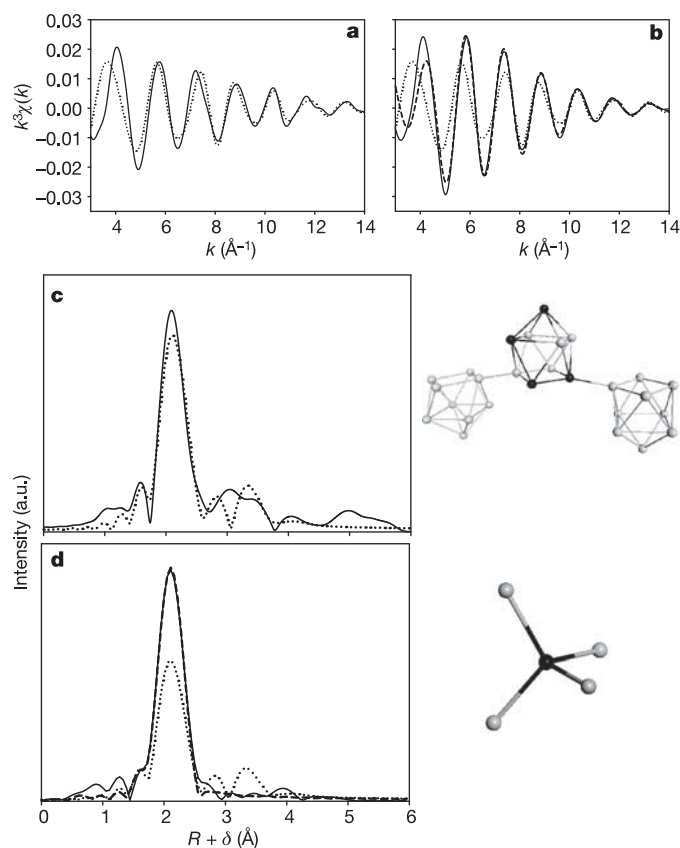


Figure 2 | EXAFS data on template Ge materials. Raw and Fourier-transformed Ge EXAFS obtained on surfactant templated germanium composites before (a and c) and after (b and d) chemical treatment. In all cases, solid lines correspond to the data, dotted lines correspond to a Zintl model generated using the cluster in c, right, and dashed lines correspond to a tetrahedral model generated using the cluster in d, right. In a and c, the Zintl cluster model fits the data reasonably well, indicating that the cage structure is retained in the hexagonal surfactant templated composite. In b and d, the data cannot be fitted with the cluster model and instead fits to the tetrahedral model. These results suggest that chemical treatment to oxidize the framework and remove the surfactant leaves the local structure similar to bulk *a*-Ge.

Because of the non-standard shape for the isotherm, however, it is important to consider whether a simple BET analysis of the data is valid. For example, the presence of micropores can cause gas adsorption in the same pressure range used for the BET analysis²⁴, producing anomalously high surface-area values. In this case, however, careful analysis of a wide range of samples suggests this is not the situation. For materials made with the optimal combination of oxidation by ferrocenium and proton treatment, BET C-constants are found to be small, BET plots are found to be linear over a large pressure range, and *t*-plots show near-zero *y*-intercepts (see Supplementary Figs S3 and S4)²⁴. Although these facts suggest minimal contributions from micropores it is important to note that the overlap of the BET and capillary condensation pressure regions could still introduce some errors into the BET surface area reported here.

One of the main goals of producing a semiconducting phase is to provide a route whereby regular porosity can be combined with size-tunable optical and electronic properties. Quantum size effects provide the main route to controlling these properties. We find that luminescence and absorption measurements on the porous materials demonstrate size-confined optical properties. Figure 3b shows luminescence spectra from two porous Ge samples. The black and the grey traces were obtained on nanoporous Ge and correspond to in-phase and quadrature (out-of-phase) signals. On the basis of precedents from the literature, the faster, in-phase signal is assigned to luminescence from localized Ge–H sites at the pore surface²⁶, whereas the longer-lifetime, quadrature signal is associated with band-edge luminescence from the Ge walls²⁷. The position of this luminescence peak is expected to shift inversely with the thickness of the Ge walls because of quantum confinement effects. Comparison of

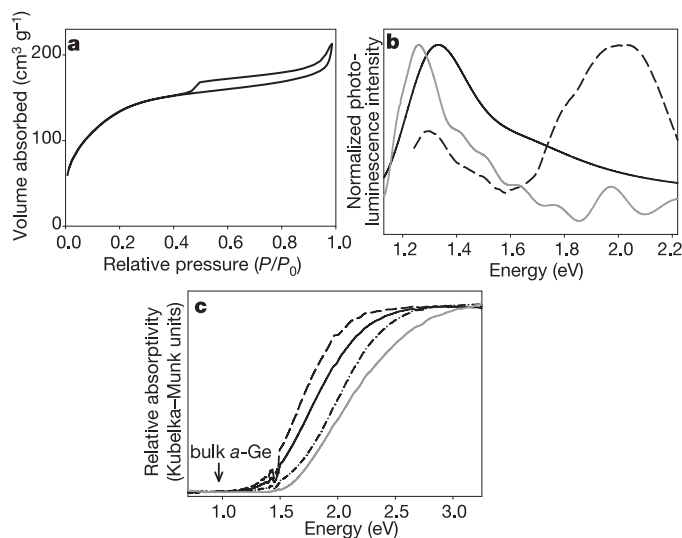


Figure 3 | Surface area and optical characterization of nanoporous Ge.

a, N_2 adsorption/desorption isotherms for mesoporous germanium obtained after chemical treatment of the hexagonal composite, showing a surface area of $500 \text{ m}^2 \text{ g}^{-1}$. **b**, Photoluminescence obtained on various nanoporous Ge materials. Black and grey solid lines correspond to in-phase and quadrature data, respectively, obtained on nanoporous Ge. The bluer in-phase peak corresponds to localized Ge–H sites, while the redder quadrature peak is consistent with $\sim 1\text{-nm}$ -thick quantum confined Ge. If the sample is oxidized in air, strong luminescence from GeO_x appears at $\sim 2 \text{ eV}$ (dashed line). **c**, Near-infrared/ultraviolet–visible reflectance. Data collected on nanoporous Ge (black) shows an extrapolated optical bandgap of $\sim 1.4 \text{ eV}$, consistent with $\sim 1\text{-nm}$ -thick quantum confined *a*-Ge. The bandgap can be shifted to the red by binding TCNE to the pore surface (dashed) or shifted to the blue by oxidizing the walls to decrease their thickness (dot-dashed). Materials with an even bluer bandgap can be produced by using a mixture of Si and Ge in the walls (grey).

peak positions to quantum-confined Ge systems with a known geometry (*a*-Ge:H/*a*-Si:H quantum wells) thus provides an estimate of the Ge wall thickness. Our luminescence peak position (1.2–1.3 eV), indicates a Ge wall thickness of ~ 1 nm (ref. 27). This value is reasonable given the position of the low-angle diffraction peaks, the size of the surfactant used, and the wall thicknesses observed in related silica-based systems.

If the sample is exposed to oxygen (Fig. 3b, dashed curve), the luminescence spectra changes dramatically. The strong luminescence peak at 2.0 eV can be related to GeO_x defects²⁸, whereas the peak in the near infrared is again assigned to the *a*-Ge network itself. Such GeO_x defects are highly luminescent and tend to dominate any spectra. The absence of any GeO_x luminescence in the nanoporous Ge confirms that these samples are well H-terminated.

Reflectance measurements can be used to calculate an optical bandgap for these materials, and the results also show quantum confinement effects similar to those seen in luminescence (Fig. 3c). The porous material (black curve) shows an extrapolated absorption onset of 1.4 eV, which again corresponds to a wall thickness of ~ 1 nm when compared to the bulk *a*-Ge band gap of 1.0 eV (ref. 27). Reflectance measurements also show how this bandgap can be tuned: for the partly oxidized sample discussed above we would expect a bluer bandgap because conversion of part of the 1-nm-thick Ge wall to GeO_x should effectively reduce the *a*-Ge wall thickness. In agreement with this idea, the entire absorption spectrum shifts to the blue.

In bulk semiconductors, surface band energies can also be shifted by binding molecules to the semiconductor surface²⁹. Because all atoms in our material are within a few angstroms of a surface, this type of binding can be used to shift the absorption. For example, tetracyanoethylene (TCNE) has a moderately low lowest-unoccupied molecular orbital (LUMO)³⁰. If TCNE is adsorbed onto nanoporous Ge, the TCNE LUMO can mix with the *a*-Ge conduction band. This mixing results in a lowering of the conduction band energy and a red-shift of the entire absorption spectra (Fig. 3c, dashed trace). We note that conductivity is exponentially dependent on the semiconductor bandgap, so a small change in bandgap should produce large changes in conductivity.

Finally, because of the flexible bonding found in Zintl clusters, the chemistry presented here can be modified to include other elements. For example, instead of starting with Ge_9 clusters, we can produce mixed Si/Ge clusters. This material can be co-organized with surfactant, ferrocenium-oxidized, and proton-treated like the pure Ge system. The result is a nanoporous Si/Ge alloy material that again shows a single peak in low-angle XRD ($q = 0.196 \text{ \AA}^{-1}$) and shows high surface area by nitrogen absorption analysis ($\sim 300 \text{ m}^2 \text{ g}^{-1}$). The bandgap of this Si/Ge material (Fig. 3c, grey trace) is significantly blue-shifted. We anticipate that various alloys of Si, Ge and Sn should allow for bandgap tunability of periodic nanoporous materials through the visible and near-infrared regions.

Our results show that it is possible to produce porous nanoscale semiconductors with a periodic, interconnected framework and a large, accessible surface area. The optical properties of these materials can be tuned by changing nanometre-scale dimension, surface-adsorbed species, or atomic composition. The results show promise for using surfactant-directed self-organization in the production of periodic porous optical and electronic materials.

METHODS

Synthesis of nanoporous Ge and Ge/Si. Germanium Zintl phases (both Ge and mixed Ge/Si) were synthesized from nominal ratios of elemental germanium (silicon) and potassium in a closed niobium vessel under argon atmosphere. Solids were heated at 700 °C for two days. In a typical nanocomposite synthesis, 0.3 g of solid K_2Ge_9 was dissolved in 10.0 ml of distilled ethylenediamine. The olive-green supernatant containing $(\text{Ge}_9^{2-})_n$ was then mixed with 3.30 g of CTEAB surfactant dissolved in 50.0 ml of distilled ethylenediamine. For a typical oxidation reaction, 0.08 g of ferrocenium hexafluorophosphate in 5.0 ml acetonitrile was added dropwise to the composite (0.24 g) in ethylenediamine under vigorous stirring. The mixture was stirred for 0.5–1 h and filtered under inert

atmosphere. The dark solid was washed twice with 10.0 ml of acetonitrile. For proton treatment, the oxidized composite (0.16 g) was suspended in 20.0 ml formamide along with 2.0 g of proton sponge (Acros, Amberlyst 15 dry ion-exchange resin). This mixture was held at 90 °C for ~ 12 h. The red-brown solid was syringed out, filtered, and dispersed in 20.0 ml of acetonitrile. This mixture was kept at 60 °C overnight before filtration. Although the $(\text{Ge}_9^{2-})_n$ polymer is not stable in formamide, after oxidation with ferrocenium, little additional reaction occurs between the composite and the formamide. The final porous Ge was washed with acetonitrile; typical yields were $\sim 35\%$. To ensure good hydrogen surface passivation, some samples were washed with a 5% HF aqueous solution and rinsed with dried ethanol. The synthesis of the mesoporous Ge/Si followed the same procedure used for the nanoporous germanium, starting with 0.5 g of $\text{K}_4\text{Ge}_5\text{Si}_4$ and 3.7 g CTEAB.

Materials characterization. Low-angle XRD of the composites was collected using a Rigaku UltraX18 rotating-anode X-ray source ($\lambda = 0.711 \text{ \AA}$), equipped with a Roper Scientific 1242 $\times$ 1152 pixel cooled X-ray charge-coupled device (CCD) detector. EXAFS data was collected on beamlines 4-1 and 6-2 at the Stanford Synchrotron Radiation Laboratory. Data was collected in transmission geometry at temperatures below 100 K using either a liquid nitrogen or liquid helium cryostat. Data analysis was performed using EXAFSPAK (<http://www-ssrl.slac.stanford.edu/exafspak.html>) and FEFF 7 (<http://leonardo.phys.washington.edu/feff/>). Nitrogen adsorption data was collected using a Micromeritics ASAP 2010 porosimeter. Elemental analysis made use of energy dispersive spectroscopy X-ray analysis on a JEOL TSM-6700F field emission scanning electron microscope equipped with a liquid-nitrogen-cooled EDAX Super UTW Detector. Data was obtained at 15 keV and 20 mA. TEM images were collected on a JEOL 2000FX microscope operated at 200 kV. XPS data was obtained on a Kratos Axis Ultra system using Al K α radiation. Powder sample were compressed onto conductive carbon tape and loaded under inert conditions.

Reflectance ultraviolet/visual-infrared data was collected at room temperature using a Shimadzu UV-3100 spectrophotometer with an ISR-3100 integrating sphere. For fluorescence measurements, samples were excited with 30 mW of light at 532 nm. Lock-in detection with a modulation frequency of 365 Hz was used to generate the in-phase and quadrature signals. All spectra were collected at 77 K.

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Subcontinental-scale crustal velocity changes along the Pacific–North America plate boundary

J. L. Davis¹, B. P. Wernicke², S. Bisnath¹, N. A. Niemi^{2†} & P. Elósegui^{1†}

Transient tectonic deformation has long been noted within ~100 km of plate boundary fault zones and within active volcanic regions, but it is unknown whether transient motions also occur at larger scales within plates. Relatively localized transients are known to occur as both seismic and episodic aseismic events¹, and are generally ascribed to motions of magma bodies, aseismic creep on faults, or elastic or viscoelastic effects associated with earthquakes. However, triggering phenomena^{2,3} and systematic patterns of seismic strain release at subcontinental (~1,000 km) scale along diffuse plate boundaries^{4,5} have long suggested that energy transfer occurs at larger scale. Such transfer appears to occur by the interaction of stresses induced by surface wave propagation and magma or groundwater in the crust⁶, or from large-scale stress diffusion within the oceanic mantle in the decades following clusters of great earthquakes⁷. Here we report geodetic evidence for a coherent, subcontinental-scale change in tectonic velocity along a diffuse ~1,000-km-wide deformation zone. Our observations are derived from continuous GPS (Global Positioning System) data collected over the past decade across the Basin and Range province, which absorbs approximately 25 per cent of Pacific–North America relative plate motion. The observed

changes in site velocity define a sharp boundary near the centre of the province oriented roughly parallel to the north-northwest relative plate motion vector. We show that sites to the west of this boundary slowed relative to sites east of it by ~1 mm yr⁻¹ starting in late 1999.

The Basin and Range Geodetic Network (BARGEN) is the first 1,000-km-aperture continuous GPS network to be deployed across a diffuse actively deforming plate boundary zone. Eighteen sites, comprising an east–west transect between latitudes 39°N and 41°N (Fig. 1), began recording in 1996–7. The time series are now long enough to obtain statistically reliable estimates of any changes in velocity that might occur on a regional scale. Dual-frequency GPS phase data from 1996.5–2005.7 were first analysed using the GAMIT/GLOBK GPS processing software, resulting in estimated average velocities and time series in a North American reference frame, as described in the Methods section. The average horizontal velocities of the sites (Fig. 1a) rise from near zero in the east to ~3 mm yr⁻¹ due west across western Utah, remain relatively constant across eastern Nevada, and then rotate northwestward and progressively increase up to ~12 mm yr⁻¹ in the Sierra Nevada.

Analysis of the position time series involves fitting for, and removal

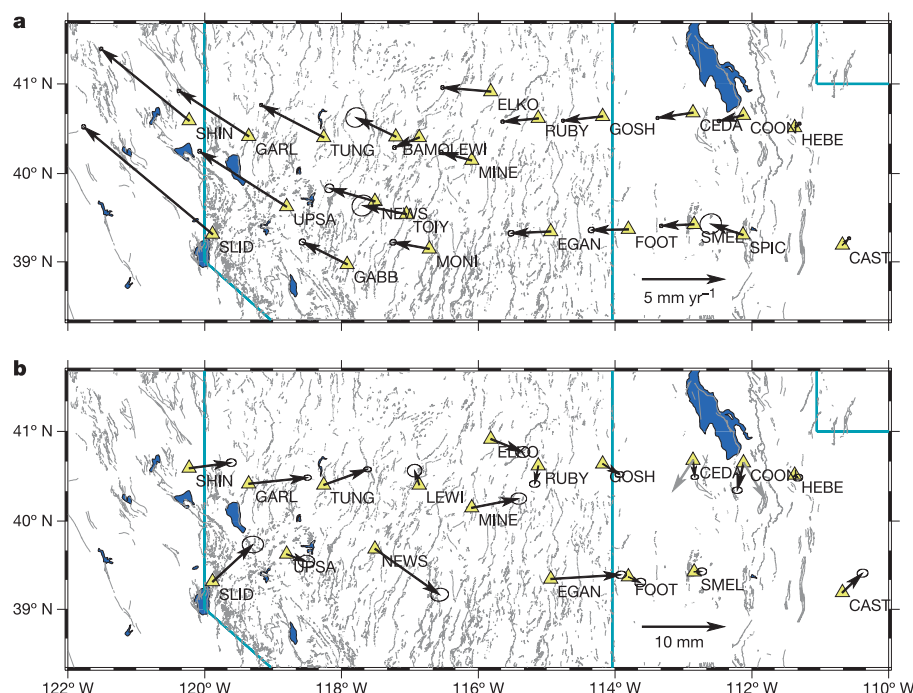


Figure 1 | Sites of the northern BARGEN GPS network. **a**, Positions (triangles) and average horizontal velocities (arrows). Error ellipses are 95% confidence (formal). Sites TOIY, BAMO, SPIC, GABB and MONI are shown, but their time series are too short to enable reliable determinations of velocity changes. Solid blue lines indicate US state boundaries. **b**, Deviations from linear motion, defined as the difference between the average position for the last year and the position predicted from a linear fit through the first 2.5 years of acquisition. Error ellipses are 95% confidence based on formal uncertainties scaled by the weighted root-mean-square residual to the linear fit and assuming the same north-east correlation as for the velocity estimates. The grey arrows for CEDA and COON represent a prediction based on a model for Great Salt Lake loading⁸.

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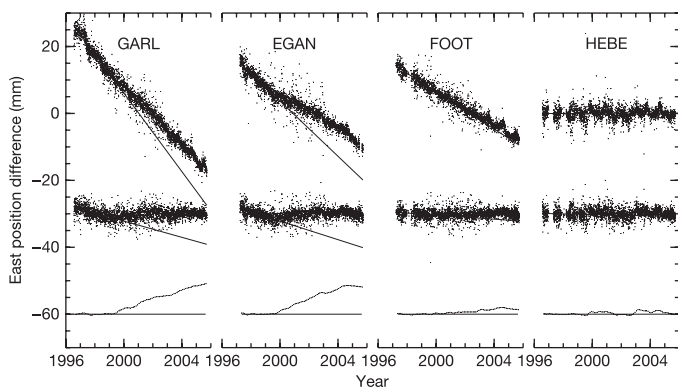


Figure 2 | Illustration of the post-analysis procedure, using time series of east position for four BARGEN sites. Top, 'raw' time series, in a North America-fixed geodetic reference frame (see Methods). Error bars are omitted for clarity, but are generally ~ 1 mm. The straight line is the best-fit straight line using position estimates from the first 2.5 years. Middle, residuals of the raw time series from a best-fit model consisting of a straight line and seasonal (annual and semi-annual sinusoids) terms. A statistical approach that allowed these terms to change with time in a constrained manner was used (see Methods). Bottom, residuals smoothed with a Gaussian filter with a full-width at half-maximum (FWHM) of ~ 8 months. A model based on a linear fit to the first 2.5 years of data has been removed. The evolution of these final time series thus indicates deviation from temporally linear motion.

of, annual variations (see Methods) and smoothing, as illustrated in Fig. 2. Using smoothed horizontal position time series, we then calculate the deviation of position from temporally linear motion based on data acquired in the first 2.5 years (hereafter referred to as 'position deviations'). This analysis indicates that the velocities in

this region have not been constant. Relative to their initial trajectories the western sites have slowed, resulting in a significant eastward position deviation (Fig. 1b). These velocity changes define two coherent domains, with an abrupt north-northwest-trending boundary in eastern Nevada (Fig. 1b). Sites west of the boundary deviate from the predicted linear motion by up to 10 mm, whereas those in easternmost Nevada and Utah have small deviations. Sites CEDA and COON, located near Great Salt Lake, exhibit large deviations due primarily to changes in lake level⁸ and possibly to hydrological loading. The estimated deviation for site SLID includes rapid deformation from 2003–4, interpreted to be the result of magma injection into the lowermost crust beneath Lake Tahoe⁹. Observed changes in velocity for the western sites are ~ 5 –10% of the respective average values.

The velocity changes are small, but they are evident in the position-deviation time series. Nearly all sites west-southwest of a boundary drawn between sites RUBY and ELKO to the north and sites FOOT and EGAN to the south have significant position deviations (Fig. 3a). With the exception of SLID, the onset of the deviation (that is, the velocity change) occurs between 1999.5 and 2001.0. The abruptness of the boundary in eastern Nevada is demonstrated by the fact that the relative position deviations across the boundary calculated by differencing sites adjacent to it are about the same as those derived by differencing sites at opposite ends of the network (Fig. 3b). If average position deviations for the western and eastern parts of the network are calculated by 'stacking' all of the position-deviation plots (Fig. 3c), the average position deviation for the west at the beginning of 2005 is ~ 5 mm, whereas that for the east is < 1 mm.

We can test this interpretation further by fitting the east components with a model consisting of two straight lines, one before 1 January 2000 and one after it. Significant nonlinearity is indicated

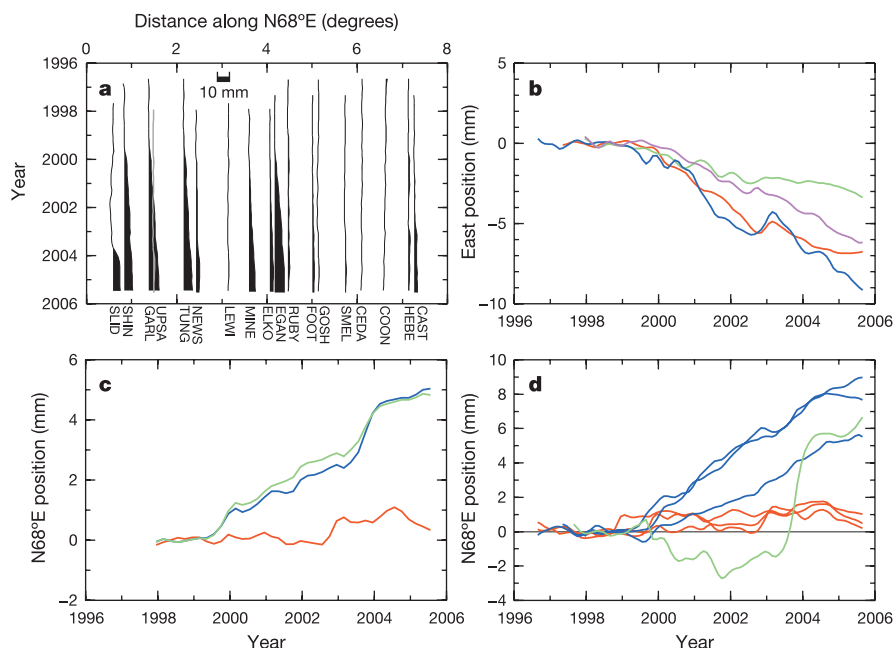


Figure 3 | Analysis of spatial variation of nonlinear deviations.

a, Smoothed time series of position deviations from linear motion (see Fig. 2) in the direction N68°E, projected along a great circle with azimuth N68°E near the centre of the network. Where these deviations are positive, the space between the trace and zero has been shaded black (or grey, for site UPSA, whose line lies atop that for GARL). The significant deviations occur in the western part of the network. **b**, East components of intersite vectors for EGAN–FOOT (red), ELKO–GOSH (green), GARL–HEBE (blue) and MINE–SMEL (purple). GARL–HEBE, which spans the entire network east–west, and EGAN–FOOT, which spans a short distance in the centre of the network (Fig. 1), show nearly identical deviations, indicating an abrupt

boundary for velocity changes in eastern Nevada. **c**, Regionally averaged nonlinear deviations of N68°E position. Red, eastern BARGEN (HEBE, FOOT, COON, CAST, CEDA, SMEL, GOSH and RUBY). Green, central/western BARGEN (MINE, TUNG, ELKO, EGAN, LEWI, NEWS, GARL, UPSA and SHIN). Blue, same as green plus SLID. Only data from a common epoch range (1997.86–2005.18) were used. The figure demonstrates that the velocity change has moved the western part of the network, on average, 3–4 mm eastward or northeastward compared to the eastern part of the network. **d**, N68°E position time series for three groups of sites. Red, eastern sites RUBY, FOOT and HEBE. Blue, western sites EGAN, MINE and GARL. Green, site SLID.

if the F-statistic¹⁰ exceeds a limit. All sites except CEDA, COON, FOOT, HEBE and LEWI pass this test for nonlinearity with 99.9% confidence. However, this test is sensitive to the degrees of freedom in the solution, which can be fewer than the assumed value if the noise in the data is temporally correlated. If we assume a correlation time of ~ 1 week, then sites CAST, ELKO, GOSH, SMEL and UPSA also fail the nonlinearity test. This larger list of 'linear' sites includes all those east of the RUBY–FOOT–ELKO line. Among sites west of this line, only UPSA and LEWI fail the F-test for nonlinearity.

There may be non-tectonic contributions to the observed deviations. Shortcomings in models used to analyse the GPS data could induce correlated systematic errors in the position estimates. The most obvious candidates are probably insignificant compared to the observed effects. Simulations of satellite antenna phase-offset and phase-pattern¹¹ effects indicate that in this region these errors are ≤ 0.2 mm. Second-order ionospheric effects may be at the millimetre level. This error depends on magnetic latitude¹², which varies by only $\sim 2^\circ$ over this network, and is therefore very nearly homogeneous for the network. Reference-frame errors can also be significant, but it is unlikely that they would yield significant intra-network deformations for a network of this size.

Another possibility is that the deviations are real but do not reflect a tectonic process. For example, the crust deforms owing to hydrological and atmospheric pressure loading and movement of the local soil. However, local effects are inconsistent with the coherence of the patterns, and numerical predictions of regional pressure loading yield deviations one order of magnitude smaller than those observed.

Site velocities in central Nevada may reflect postseismic deforma-

tion of strong (magnitude $M > 6.5$) twentieth-century earthquakes (Pleasant Valley, 1915; Dixie Valley, 1954; Fairview Peak, 1954). Investigators have used GPS^{13,14} and InSAR¹⁵ to determine models for viscoelastic relaxation of the crust and upper mantle. During the short time period of this study relative to reasonable viscoelastic relaxation times, viscoelastic relaxation will not affect the position deviations.

Supporting evidence for a tectonic origin, which may also provide a clue to its depth of generation in the lithosphere, comes from the apparent lower crustal source of SLID's previously reported⁹ rapid transient in late 2003. Unlike the other Nevada sites, SLID (although noisier) did not systematically accelerate eastward through most of the observation period, being 'left behind' by the accelerating sites. Then, by virtue of the transient, it 'caught up' with the eastward deviations of the other sites (Fig. 3d). We speculate that an episodically creeping detachment horizon ~ 500 km wide at or near the base of the crust may provide a general explanation for the velocity changes. In one scenario, the mantle would either translate or stretch smoothly below the horizon, imparting a westward component of shear traction on the base of the crust (before 1997 to about 2000). Top-to-the-east plastic yielding along the horizon translates the crust eastward *en bloc*, as internal stretching and shear of the crust continues. Yielding thus relaxes stress on the eastern margin of the accelerating domain (where baselines ELKO–GOSH and EGAN–FOOT were contracting at ~ 0.5 mm yr⁻¹ and 1 mm yr⁻¹, respectively), and focuses strain accumulation (2000–3), and subsequently strain release (2003–4) along the western margin of the domain.

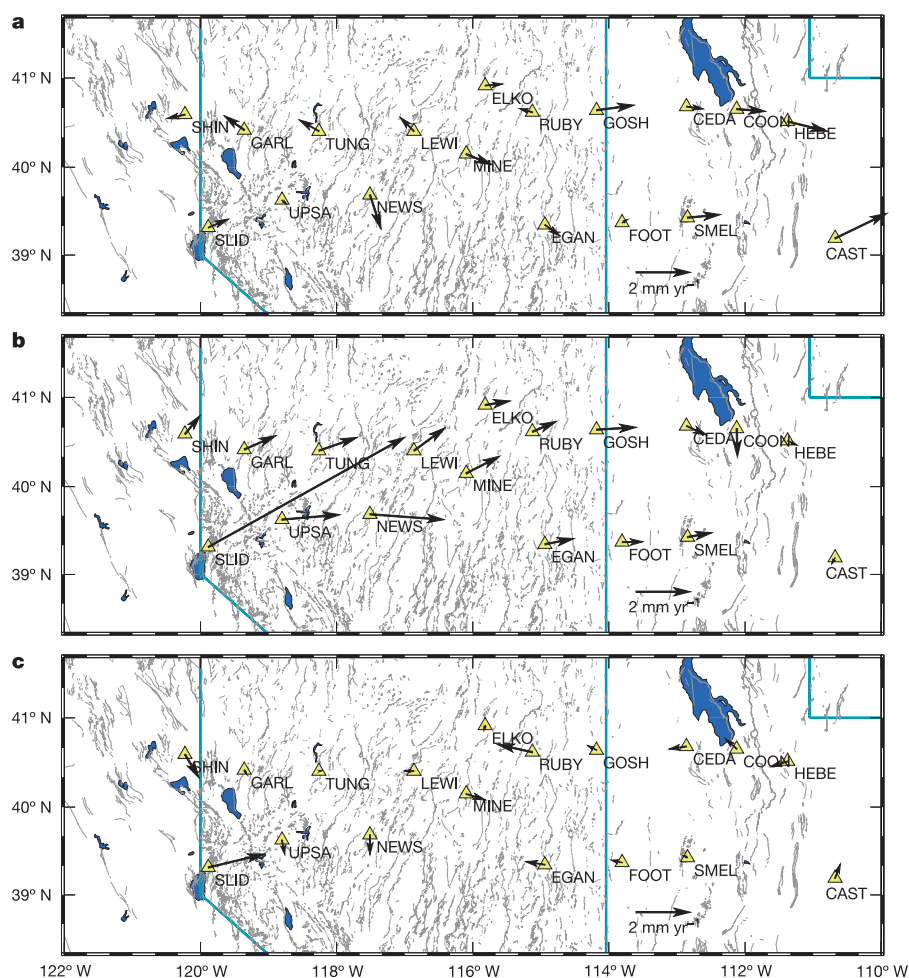


Figure 4 | Differences of horizontal velocity for one-year periods relative to the average velocities for the period 1997.0–2002.0. **a**, Differences for the calendar year 2002. **b**, 2003. **c**, 2004. 1σ errors for each component are ~ 0.4 mm yr⁻¹.

Several independent lines of evidence support a contrast in lithospheric properties between the western and eastern parts of the network. On the basis of S-wave tomography and petrologic data, Dixon *et al.*¹⁶ have proposed that the upper mantle beneath the western part of the network contains a higher concentration of partial melt. An active decoupling zone at the base of the crust under the Nevada Basin and Range, accompanied by magmatic injection, has long been suspected on the basis of nearly continuous, bright seismic reflections along the Moho^{17–19}. Like the GPS velocity change, these reflections also die out near the Nevada–Utah border, suggesting that the reflection Moho may be an active, coherent structure that episodically yields, thereby transferring strain energy hundreds of kilometres across the province.

Within the overall pattern of velocity changes that begin in about 1999.5, spatially coherent velocity changes associated with the onset of the anomalous SLID motion can be observed. Figure 4 shows the site velocity differences averaged over one-year intervals relative to the average velocities for 1997.0–2002.0 for one-year slices of the time series. The relative velocities for the year 2002.0–2003.0 (that is, calendar year 2002; Fig. 4a) are mostly $<1 \text{ mm yr}^{-1}$ and exhibit little regional coherence, except for an eastward trend for GOSH, SMEL and sites eastward. During 2003 (Fig. 4b), most of the anomalous SLID motion occurred, resulting in an apparent northeast velocity change of $\sim 9 \text{ mm yr}^{-1}$. During this year, most of the sites exhibited a smaller, easterly or northeasterly velocity change, the amplitude of which decreases to the east (Fig. 4a; see also Fig. 3c). During 2004 (Fig. 4c), all the sites returned to within $\sim 1 \text{ mm yr}^{-1}$ of their average 1997–2002 average velocities, except for SLID, which exhibited a small amount of residual motion associated with the 2003 event. For the eastern sites, a coherent westward increase in velocity of $\sim 1 \text{ mm yr}^{-1}$, relative to that of 2003, is observed. (This increase is clearly reflected in the averaged time series of Fig. 3c.) These motions are further evidence for large-scale strain transfer, with anomalous motions occurring first in the east and later propagating west on a timescale of ~ 1 year.

METHODS

Geodetic solutions for position and velocity were generated using the GAMIT/GLOBK software package, following methods previously detailed²⁰. Briefly, the dual-frequency GPS phase data were analysed on a daily basis using GAMIT. Data from some non-BARGEN GPS sites were included for frame determination. The resulting site-position parameters were combined using the Kalman-filter-based GLOBK software to provide estimates of (average) velocity vectors. We also incorporated into our solution data products from other continuous GPS networks, including the International GNSS Service (IGS) and Bay Area Regional Deformation (BARD) networks, obtained from the Scripps Orbit and Permanent Array Center (SOPAC). Minimization of velocity parameters for sites on 'stable' North America provided an approximation to a North America-fixed reference frame.

Time series were generated using the GLRED software, which minimized site position deviations on a day-by-day basis in a realization of the North America reference frame. In analysing time series of horizontal position estimates, we used a statistical approach that allowed for estimation of nuisance parameters (mainly seasonal variations) when arbitrary changes in velocity occur over the length of the time series. We first removed all apparent 'jumps' that occur owing to changes in antennas and radomes. (Only three such jumps are required for all the data presented here.) We used a technique that 'sutures' the time series together using data only from the time around the jump (± 1 month), thereby minimizing the effect of possible mismodelling of the temporal behaviour of the time series.

We then modelled the time series over one-year segments using parameters that make up the two components of the model: linear terms plus seasonal terms. The seasonal component consists of annual and semi-annual sinusoids with amplitude (sine and cosine terms) parameters. The two components of the model (linear and seasonal) were individually constrained to be piecewise continuous at the segment boundaries. The first time derivative of the seasonal component at the segment boundaries was likewise constrained. Typical formal uncertainties are $\sim 0.3 \text{ mm yr}^{-1}$ for the rates, and $\sim 0.1 \text{ mm}$ for the seasonal

amplitudes. For each site, the north and east components of position were analysed independently. We did not apply spatial filtering²¹, as this technique removes spatially correlated nonlinear signals.

This approach provides two useful products that can be used for further analysis. We obtain clean time series of site position that have the (variable) seasonal terms removed. We also obtain time series of site velocities that can be used to investigate the spatial coherence of velocity changes. These time series have been derived with no assumptions regarding the spatial variations of site position changes.

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Seismic waves increase permeability

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Earthquakes have been observed to affect hydrological systems in a variety of ways—water well levels can change dramatically, streams can become fuller and spring discharges can increase at the time of earthquakes^{1–7}. Distant earthquakes may even increase the permeability in faults⁸. Most of these hydrological observations can be explained by some form of permeability increase^{1,5}. Here we use the response of water well levels to solid Earth tides to measure permeability over a 20-year period. At the time of each of seven earthquakes in Southern California, we observe transient changes of up to 24° in the phase of the water level response to the dilatational volumetric strain of the semidiurnal tidal components of wells at the Piñon Flat Observatory in Southern California. After the earthquakes, the phase gradually returns to the background value at a rate of less than 0.1° per day. We use a model of axisymmetric flow driven by an imposed head oscillation through a single, laterally extensive, confined, homogeneous and isotropic aquifer to relate the phase response to aquifer properties⁹. We interpret the changes in phase response as due to changes in permeability. At the time of the earthquakes, the permeability at the site increases by a factor as high as three. The permeability increase depends roughly linearly on the amplitude of seismic-wave peak ground velocity in the range of 0.21–2.1 cm s⁻¹. Such permeability increases are of interest to hydrologists and oil reservoir engineers as they affect fluid flow and might determine long-term evolution of hydrological and oil-bearing systems. They may also be interesting to seismologists, as the resulting pore pressure changes can affect earthquakes by changing normal stresses on faults¹⁰.

We use the response of wells to solid Earth tidal strains as a probe of the *in situ* permeability to provide a long-term, continuous record of permeability evolution^{9,11,12}. The pressure in confined aquifers continually oscillates in response to the solid Earth tide. The volumetric strain of the Earth tide increases the pore pressure in the confined aquifer. Therefore, a pressure gradient is generated and the water flows in and out of the well, producing oscillations in water level. If the permeability of the aquifer is high, then the oscillations in the well are nearly in phase with the imposed tidal strain. If the permeability is low, then the water level oscillations lag significantly behind the solid Earth tidal strain as it takes some time for water to flow into the well. In between the extremes, there is a finite phase lag of the tidal oscillations in the well relative to the imposed tidal strain. The phase lags provide a means of measuring permeability.

In this study we consider water level data (Fig. 1) from two water wells in fractured granodiorite at Piñon Flat Observatory (PFO) in southern California (33.610° N, 116.457° W)¹³. The wells (CIB and CIC) are 300 m apart, and were drilled in 1981 to depths of 211 m and 137 m, respectively. Both are cased to 61 m and neither is pumped. Figure 1 shows the long-term water-level changes in these wells, driven in part by local precipitation; the inset shows the oscillations from the Earth tides. The dashed lines in Fig. 1 show the times of earthquakes (Fig. 2) that produced large shaking (see legend of Fig. 1).

We measure the phase shift of the water level relative to the dilatational strain for the semidiurnal tides (See Methods and Supplementary Information). Negative phases imply greater delay, so we refer to less negative phase shifts as smaller phase lags. As discussed above, the smallest lags (closest to zero) imply the highest permeabilities in the system.

Transient changes in the phase response are plainly seen in Fig. 3 at the times of all the earthquakes (dotted lines). Each transient change is characterized by a step in the phase response at the time of the earthquake followed by a gradual recovery of the phase to its pre-earthquake value. The phases recover linearly with time, though with different rates for the two wells: 0.08° per day for CIB and 0.04° per day for CIC.

We also evaluated the amplitude of the tidal response, which shows much smaller variations, especially in well CIC (see Supplementary

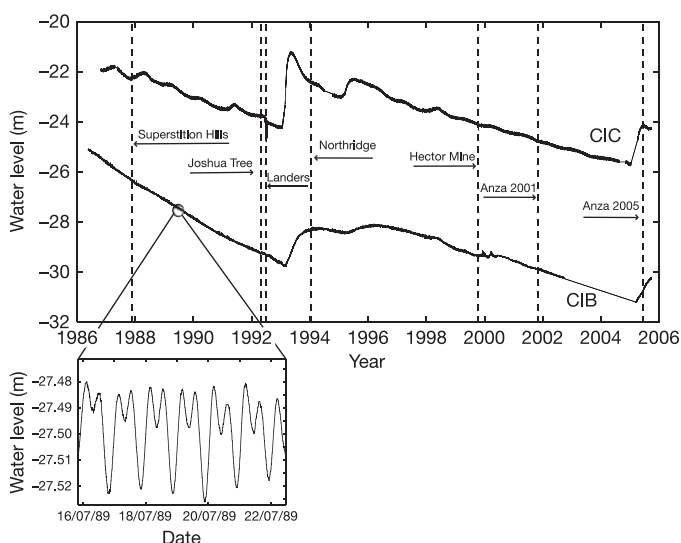


Figure 1 | Water level from wells CIB and CIC. Vertical dashed lines show the times of all earthquakes in the SCEC earthquake catalogue for Southern California (defined as 32° N–35° N; 119° W–114° W) with $M_L \geq 3$ that produced a synthetic Wood–Anderson amplitude of 1 m (~ 0.3 mm true ground motion at 1 Hz). This arbitrary threshold was chosen to provide a way to select earthquakes with large shaking systematically, because continuous on-scale seismic recording was not available at PFO for the entire study period. The set of seven earthquakes, which excludes aftershocks occurring within a month of the largest event, is robust to small differences in the M_L –distance relationship and the selection of earthquakes does not change even if the Wood–Anderson amplitude threshold is lowered to 0.5 m. Amplitudes are calculated from the magnitude–distance relationship of ref. 21. Long-term water-level changes are driven by local weather and seasonal rainfall. The oscillations in the inset are generated by Earth tides.

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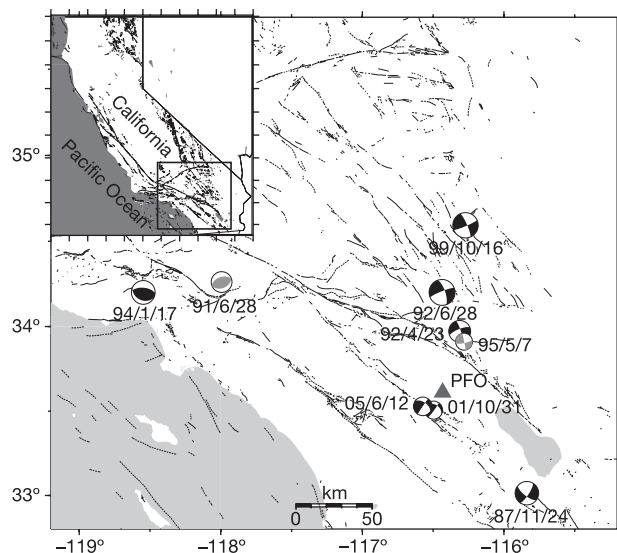


Figure 2 | Map of Southern California, showing epicentres of earthquakes considered in this study, as selected by the criterion in Fig. 1. The focal mechanisms of most earthquakes were obtained from the Hardebeck and Shearer catalogue²². The focal mechanisms of the Landers 92/6/28, Hector Mine 99/10/16 and Anza 05/6/12 earthquakes were obtained from the Harvard CMT catalogue because the Hardebeck and Shearer catalogue²² does not include large events (Landers $M_w = 7.3$ and Hector Mine $M_w = 7.1$) or the latest earthquakes such as the Anza 2005 quake. The relative size of the beach ball symbols corresponds to the relative magnitudes of the earthquakes; 4.8 for the smallest and 7.3 for the largest. (For more information please see Supplementary Table S1.) Piñon Flat Observatory (PFO) is shown by a triangle. The light-shaded focal mechanisms show the two additional earthquakes described in the legend of Fig. 3.

Information). The amplitude changes are relatively easily mapped into small storage changes and, unlike the phase, they are nearly insensitive to the permeability changes.

The observed changes at the times of the earthquakes imply that some earthquake-induced stress affected these well-aquifer systems. The change in static stress field and the dynamic stress from the seismic waves are both candidates. The static stress change for areal strain has the same sign as the first motion on seismograms, and for these earthquakes the first motions at PFO include both extension and compression. This is supported by calculations of static fields at PFO, which also show compression for Northridge and Anza 2001, but extension for all other earthquakes (Supplementary Table S1). But all earthquakes produced a decrease in the phase lag. Regardless of the detailed hydrological model, a phase lag decrease corresponds to an increase in permeability, which is unlikely to be produced by local compression and is extremely unlikely to be caused by both extension and compression. The dynamic, rather than static, strains are the probable cause, and we use the vertical peak ground velocity (PGV) as a proxy for the dynamic strain field¹⁴ (Fig. 2). We measure the PGV directly from seismic records at PFO. The phase change is the difference between two measurements made before and after each earthquake over 200-h windows (see Supplementary Figs S3 and S4).

We quantitatively interpret aquifer phase lags as permeability (see the Methods section), following the methods of refs 9 and 3. Figure 4 plots the inferred changes in permeability at the time of the earthquakes against the PGV. For PGV values above 0.2 cm s^{-1} , the data show a linear relationship between the permeability change and the ground shaking:

$$\Delta k = R \frac{v}{c} \quad (1)$$

where Δk is the change in permeability at the time of the earthquake, v is PGV in the vertical and c is the phase velocity of the seismic

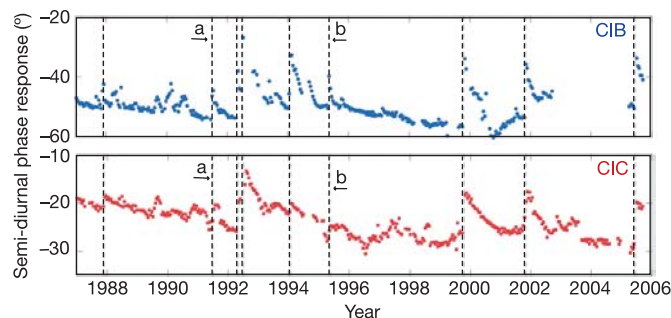


Figure 3 | Phase of the semidiurnal tide for the water level in the wells CIB and CIC, relative to the areal strain. Transient changes in the phase response are clearly shown at the time of the earthquakes (indicated as in Fig. 1) selected by the criterion in Fig. 1. Because our selection criterion is based on computed ground shaking at PFO, it missed at least two earthquakes: 'a' shows the 1991 Sierra Madre earthquake (PGV = 0.16 cm s^{-1}); and 'b' shows the 1995 Joshua Tree earthquake (PGV = 0.42 cm s^{-1}). These two additional earthquakes do not substantially affect the linear relationship between the permeability changes and the ground shaking defined by the set of seven earthquakes; see Fig. 4.

waves. The ratio v/c is approximately the imposed strain on the system¹⁴, so R measures the permeability response to strain. The parameter R is a property of the well-aquifer system and is different for each well. Assuming c to be 3 km s^{-1} , $R_{\text{CIB}} \approx 3.0 \times 10^{-10} \text{ m}^2$ and $R_{\text{CIC}} \approx 8.4 \times 10^{-10} \text{ m}^2$.

We derived this relationship before the 2005 Anza earthquake, which generated the largest shaking at PFO yet recorded. This example provided a good opportunity to test equation (1). As can be seen in Fig. 4, the resulting permeability changes followed the linear trend previously defined by the other data points.

A commonly reported hydrological response to earthquakes is a drop in water level^{3,4,5,11,15,16}. These Piñon Flat wells show water level drops for four of our study earthquakes. A key, unresolved problem is the relationship between these drops and the permeability enhancement recorded by the tidal phase change. We note that the tidal phase and the water level are sensitive to different regions of the well-aquifer system. The tidal phase averages the properties over an effective volume extending a distance of the order of $\sqrt{\kappa\tau}$ from the well, where κ is the hydrologic diffusivity (equal to the ratio of transmissivity and storage) and τ is the tidal period. For the hydraulic diffusivities that we infer from the PFO wells and the semidiurnal tidal period, this distance is of the order of 200 m. The two wells are 300 m apart, which is consistent with differing responses. On the other hand, persistent water level drops can record localized changes. Because of this difference, we are not surprised that the tidal response changes reveal a much more systematic relationship to PGV than is observed for water level changes in these wells.

To the best of our knowledge, the tidal responses measured here provide the first published long-term monitoring of permeability in a natural system. It is the first direct measurement of well-defined permeability increases in a natural setting before and after multiple earthquakes at the same site. It is also the first definitive relationship between measured permeability increases and other measured stresses in the field. The measurements led to a new and, to us, surprising conclusion. Permeability in a fractured rock system was increased significantly by the small stresses in seismic waves from regional earthquakes. The peak dynamic stress σ can be estimated from the PGV using the relationship $\sigma = \mu v/c$ where v is PGV, c is the phase velocity (as above) and μ is the shear modulus ($\sim 3 \times 10^{10} \text{ Pa}$), giving values in the range of 0.02 to 0.21 MPa for PGV in the range 0.2 to 2.1 cm s^{-1} (Fig. 2). (Previous studies¹⁷ in intact laboratory samples had suggested that stresses of the order of 100 MPa were necessary to cause the large changes in the permeability that we observe.) The increases are systematic and even predictable. There is a roughly

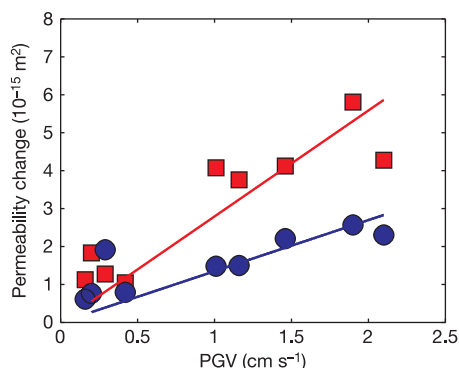


Figure 4 | Change in permeability for the well-aquifer systems CIB (circles) and CIC (squares) at the time of each of the earthquakes, plotted against peak ground velocity measured at PFO. Ground motion is low-passed at 0.5 Hz to make a comparable measurement on a variety of strong-motion and broadband seismometers. There is a linear relationship between the increase in permeability and the ground shaking for 0.2 to 2.1 cm s⁻¹ peak ground velocities. We make a linear fit that goes through the origin to be consistent with the condition of no change in permeability in the absence of shaking. The slopes of the fit as described by equation (1) are $R_{CIB} = 3.4 \times 10^{-10} \text{ m}^2$ and $R_{CIC} = 8.4 \times 10^{-10} \text{ m}^2$. With the point for CIB from the Northridge earthquake removed, the goodness of fit r^2 is 0.7 (97% significance); with it included, r^2 for CIB is 0.3 and for CIC r^2 is 0.7 (see Supplementary Fig. S4). The average background permeability values are around $1.0 \times 10^{-15} \text{ m}^2$ for CIB and $6.0 \times 10^{-15} \text{ m}^2$ for CIC, giving a maximum increase by a factor of three for CIB and by a factor of two for CIC for the largest shaking.

linear relationship between the PGV and the permeability change at each well for the range of measured PGVs.

This result has potentially far-reaching consequences. First, we have demonstrated the utility of a simple, non-invasive method for monitoring permeability in confined aquifers or reservoirs. Second, the data indicate that relatively small dynamic stresses can double or triple permeability and therefore suggests a possible method for active permeability enhancement in economically useful geothermal, natural gas and oil reservoirs¹⁸. Third, the large variations of permeability over time indicate that natural permeability is not a fixed quantity, but rather an ever-evolving, dynamically controlled parameter. Fourth, and most speculatively, the fractures and flow resulting from such permeability-enhancement processes in faults might be a stage in the dynamic triggering of earthquakes^{5,10}.

METHODS

Tidal response. The phase and amplitude tidal responses were estimated using two separate methods which yielded nearly identical results. The measurements in Fig. 3 were derived using a least-squares fit of the observed water level data to the predicted tides based on a high-precision ephemeris (see Supplementary Information). As a check, we performed a second analysis in the frequency domain by dividing the Fourier transform of the observed tide and a synthetic tide, and taking the result at the frequency of the largest semidiurnal tide (M_2). The permeability changes are robust and the results are indistinguishable from the time-domain calculation.

Flow model. In estimating aquifer properties, we follow ref. 9 in modelling the tidal response as a result of flow in a single, laterally extensive, confined, homogeneous and isotropic aquifer. In an isotropic system, the far-field tidal head oscillation is proportional to the volumetric strain. In reality, it is unlikely that the aquifer is either homogeneous or isotropic. The most important omitted effect is the coupling of shear stresses to pore pressure by anisotropic fractures¹⁹. This complication would introduce a phase shift to all of our measurements and therefore bias our permeability estimates; however, the imposed phase shift should not change over time. The relative measurements we make using the Hsieh model⁹ should be robust to these complications.

We corrected for the water table effect on the phase shift³ by adding 15° to the observed lags. The semi-confined aquifer leaks, so this correction is necessary to account for the small amount of pore pressure diffusion to the free surface. The diffusion time of the leakage is known from the recovery time of water level

drops after earthquakes. Once the diffusion time is known, we can calculate the leakage following ref. 3.

The well response depends on the flow of water through the porous medium and therefore is sensitive to the aquifer transmissivity and storage. Transmissivity is the rate of water transmission through a unit width of aquifer under a unit hydraulic gradient and is directly proportional to permeability. Storage is the strain change per unit imposed head and is a measure of compressibility.

Additional factors in the response in the most general case also include the well geometry, the period of the oscillation and inertial effects. The inertial effects are negligible for the long periods of the Earth tides²⁰. The other two factors are independently well constrained.

The amplitude A and phase η responses for the long periods of tidal oscillations are⁹:

$$A = (E^2 + F^2)^{-1/2} \quad (2)$$

$$\eta = -\tan^{-1}(F/E) \quad (3)$$

where

$$E \approx 1 - \frac{\omega r_w^2}{2T} \text{Kei}(\alpha), \quad F \approx \frac{\omega r_c^2}{2T} \text{Ker}(\alpha), \quad \alpha = \left(\frac{\omega S}{T}\right)^{1/2} r_w \quad (4)$$

and T is the transmissivity, S the dimensionless storage coefficient, Ker and Kei the zeroth-order Kelvin functions, r_w is the radius of the well (8.8 cm for CIB, 9.1 cm for CIC), r_c is the inner radius of the casing (7.9 cm for both wells) and ω is the frequency of the tide.

We use the measured phase and the amplitude responses η and A with equations (2) and (3) to solve for storage and transmissivity. The storage shows only small (<40%) changes as a function of time. This result is a direct consequence of the lack of large variations in the amplitude response.

The relationship between transmissivity and permeability is:

$$k = \frac{\mu}{\rho g d} T \quad (5)$$

where k is the permeability, μ is the dynamic viscosity, ρ is the density, g is the gravitational acceleration and d is aquifer thickness. Because none of the other parameters are likely to change during an earthquake, changes in transmissivity are interpretable as changes in permeability. The values used are $\mu = 10^{-3} \text{ Pa s}$, $\rho = 10^3 \text{ kg m}^{-3}$, $g = 9.8 \text{ m s}^{-2}$ and $d = 150 \text{ m}$.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Diversity and dispersal interactively affect predictability of ecosystem function

Kristin E. France¹ & J. Emmett Duffy¹

Theory and small-scale experiments predict that biodiversity losses can decrease the magnitude and stability of ecosystem services such as production and nutrient cycling^{1,2}. Most of this research, however, has been isolated from the immigration and emigration (dispersal) processes that create and maintain diversity in nature^{3–5}. As common anthropogenic drivers of biodiversity change—such as habitat fragmentation, species introductions and climate change—are mediated by these understudied processes^{5–7}, it is unclear how environmental degradation will affect ecosystem services^{3,4}. Here we tested the interactive effects of mobile grazer diversity and dispersal on the magnitude and stability of ecosystem properties in experimental seagrass communities that were either isolated or connected by dispersal corridors. We show that, contrary to theoretical predictions^{2,8–11}, increasing the number of mobile grazer species in these metacommunities increased the spatial and temporal variability of primary and secondary production. Moreover, allowing grazers to move among and select patches reduced diversity effects on production. Finally, effects of diversity on stability differed qualitatively between patch and metacommunity scales. Our results indicate that declining biodiversity and habitat fragmentation synergistically influence the predictability of ecosystem functioning.

Broadening the spatial scope of biodiversity–ecosystem–functioning (BD–EF) research to metacommunities—that is, groups of patches connected by dispersal of organisms—adds two components of diversity: beta-diversity, or heterogeneity in species composition among patches, and gamma-diversity, or diversity of the entire metacommunity¹². Limited evidence indicates that, at these broader spatial scales, the functional consequences of diversity may be different^{13,14}. Furthermore, the effects of dispersal among patches on ecosystem properties have rarely been considered, despite the demonstrated importance of dispersal in maintaining diversity, particularly in fragmented habitats^{5,10,15}. Given recent predictions that the mechanism of diversity maintenance strongly influences relationships between biodiversity and ecosystem function^{15,16}, and the increasingly fragmented character of most habitats, scaling up BD–EF research to metacommunities is critical for its application to conservation.

Here we test how diversity, dispersal and spatial scale interactively affect properties of experimental multitrophic seagrass (*Zostera marina*) ecosystems. We assembled metacommunities with low (three spp.) and high (eight spp.) grazer species richness, simulating loss of rare species from a species pool. Each metacommunity had five patches that were either interconnected by dispersal corridors or unconnected (see Methods). Dispersal was extremely rare among unconnected patches and moderate among connected patches. Each patch received 30 mobile crustacean grazers (15 male–female species pairs), and the species composition of this founding community was determined by random draws from the appropriate species list (three

versus eight spp.). Initial metacommunity-wide richness of grazers was set at either three or eight species, but both the relative abundances of species within metacommunities and the species richness within patches varied. We allowed this initial random assembly plus subsequent dispersal and species interactions to influence grazer diversity over the six-week-long experiment. This enabled us to determine how multiple spatial components of biodiversity affect ecosystem properties in both connected and unconnected metacommunities.

We tested four hypotheses. (1) Increasing metacommunity richness will increase mean patch richness^{15,17} and beta-diversity of grazers¹⁸. (2) Allowing dispersal will increase mean patch richness^{5,15} and decrease beta-diversity¹⁷ of grazers. On the basis of these predicted diversity patterns, and on previously documented links between diversity and ecosystem properties¹, we expected that (3) grazer abundance and grazing pressure will increase with metacommunity richness and dispersal^{13,15–17}. As increasing richness often increases the predictability of ecosystem properties^{2,8–11}, even in multitrophic systems¹⁹ and in metacommunities¹⁵, we also hypothesized that (4) increasing metacommunity richness should increase predictability of ecosystem properties among and within patches.

Both metacommunity richness and dispersal of grazers influenced grazer diversity (Fig. 1a–c). As predicted, increasing initial metacommunity richness increased final grazer diversity at all scales: within patches (alpha-diversity), between patches (beta-diversity), and within entire metacommunities (gamma-diversity)^{15,17}. Grazer dispersal increased compositional similarity among patches, decreasing beta- and gamma-diversity without affecting alpha-diversity in the patches (Fig. 1a–c). Both local extinctions and colonizations occurred, at varying rates for different species (Supplementary Table S1). Dispersal was frequent enough that all but one grazer species successfully founded populations in patches where they were not initially present, colonizing 25–100% of such patches. But dispersal was not so frequent that it erased the stamp of initial composition, as the final proportional abundances of most species were significantly predicted by initial proportional abundances (Supplementary Table S1). This evidence of colonization, extinction and moderate dispersal rates confirms that our connected patches functioned as true metacommunities. The effects of this active dispersal on diversity in our experiment parallel the effects of passive dispersal observed for protozoans and other zooplankton^{20,21}, indicating that the dispersal effects on metacommunity diversity we found may be robust.

Metacommunity richness and dispersal of grazers also affected net production at multiple trophic levels. Mean grazer abundance increased with grazer richness, both within patches (Supplementary Table S2) and in entire metacommunities (Fig. 2a), as predicted^{13,15–17}. Concomitantly, the larger populations of grazers in richer metacommunities more effectively cropped biomass of primary producers,

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including macroalgae, cyanobacteria, the foundation species (*Zostera*), and its epiphytic algae (Fig. 2c–f; Supplementary Table S2). These metacommunity-richness effects cannot be explained by ‘selection effects’ (that is, presence of a particular species at higher diversity), because several species from both small and large pools significantly contributed to these trends (see Supplementary Methods). Thus, even in a system with random assembly, immigration and emigration, species richness pervasively influenced community and ecosystem properties.

Compared with diversity, grazer dispersal had relatively modest effects on ecosystem properties. Contrary to our prediction^{5,15}, dispersal did not increase mean grazer diversity or abundance within patches (Fig. 1a–c, Fig. 2b). Nonetheless, dispersal did affect grazing impact, allowing grazers to actively seek patches with preferred food and abandon patches with undesirable food. Specifically, connecting patches decreased the biomass of edible macroalgae and recruits of the tunicate *Molgula manhattensis* (Fig. 2c, d). In contrast, dispersal increased the biomass of less preferred cyanobacteria and *Z. marina*, at least in the less diverse communities (Fig. 2e, f). During the first 28 days of the experiment, this dispersal-mediated shift in grazing impact actually enhanced epiphyte biomass accumulation within connected patches (Supplementary Table S2). Furthermore, connecting patches reduced the enhancement of secondary production by diversity seen in isolated patches. Specifically, total grazer abundance increased with grazer diversity, but the slope of this relationship was reduced by grazer dispersal (Fig. 2b; Supplementary Table S3). In the

absence of dispersal, diversity led to more effective and thorough grazing; however, when grazers could actively select favourable patches, they may have limited their own population growth by indirectly facilitating the colonization of limited substrate by less palatable algae²². Hence, active dispersal and habitat selection by multiple generations of grazers can affect ecosystem properties and modify the effects of biodiversity on ecosystem properties, underscoring a key difference between assemblages with single versus multiple trophic levels.

Also contrary to our expectations^{2,8–11}, higher metacommunity richness increased ecosystem variability both among and within patches. Although the similarity hypothesis predicts that increasing diversity increases compositional similarity, thereby increasing the predictability of ecosystem function across space¹¹, we found that more diverse grazer metacommunities produced greater spatial variability in ecosystem properties, including algal and sessile invertebrate biomass accumulation (Fig. 3b–d). This increased spatial variability probably stemmed from variability in grazer community composition (increased beta-diversity) in the more diverse metacommunities (Fig. 1b), supporting the hypothesis that compositional similarity and spatial predictability of ecosystem function are positively related¹¹. However, these results also indicate that when

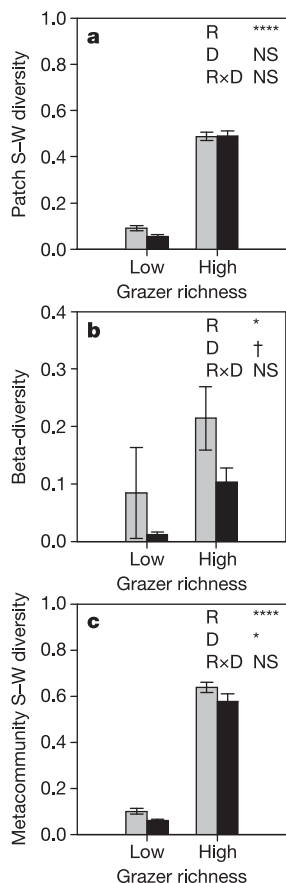


Figure 1 | Species pool size affects grazer diversity at multiple spatial scales. Grey bars indicate no dispersal corridors; black bars indicate dispersal corridors. **a**, Mean ($\pm$ s.e.m.) patch diversity (Shannon–Weaver (S–W); $n = 25$ for each bar). **b**, Beta-diversity (see Supplementary Methods (Diversity Measures); $n = 5$ for each bar). **c**, Metacommunity S–W diversity ($n = 5$ for each bar). R, richness; D, dispersal; †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; NS, not significant.

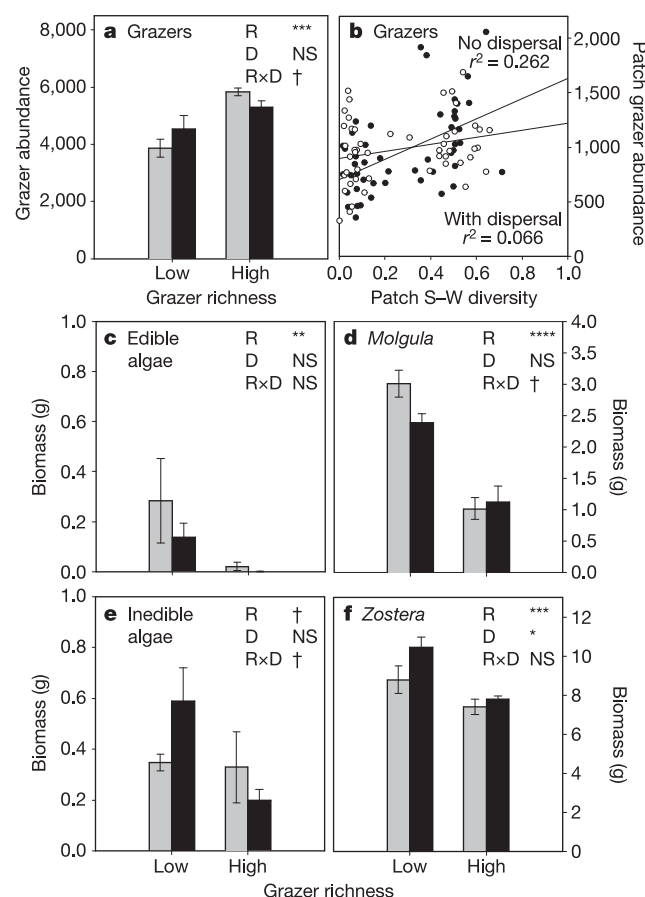


Figure 2 | Metacommunity richness and dispersal affect the magnitude of ecosystem properties at multiple trophic levels. **a**, **b**, Mean ($\pm$ s.e.m.) grazer abundance increased with diversity (**a**), but this relationship was modified by dispersal (**b**). In **b**, open symbols indicate no dispersal; closed symbols indicate dispersal. For each set, $n = 50$. **c–f**, More diverse (and denser) grazer communities more effectively reduced biomass of edible macroalgae (**c**), *M. manhattensis* recruits (**d**), less edible cyanobacteria (**e**), and *Z. marina* (the foundation species) (**f**). Connecting patches allowed grazers to concentrate on preferred food (**c**, **d**), facilitating accumulation of less preferred food (**e**, **f**). For each bar, $n = 5$. Symbols are as in Fig. 1.

species are lost from entire landscapes—and not just local communities—declining diversity may increase patch compositional similarity, producing a negative relationship between diversity and predictability in space. The contrast between our results and previous experiments^{11,23,24} highlights the importance of examining how different biodiversity-loss scenarios affect ecosystem function, and recognizing that higher diversity does not necessarily increase the predictability of ecosystem functioning in space as it often does in time.

Metacommunity grazer richness also affected temporal variability of ecosystem properties, but in surprising, scale- and dispersal-dependent ways. Temporal variabilities of both grazer abundance and epiphyte load were generally lower in metacommunities than in patches (Fig. 3e–h). At the patch scale, increasing grazer richness increased temporal variability of grazer abundance (Fig. 3e),

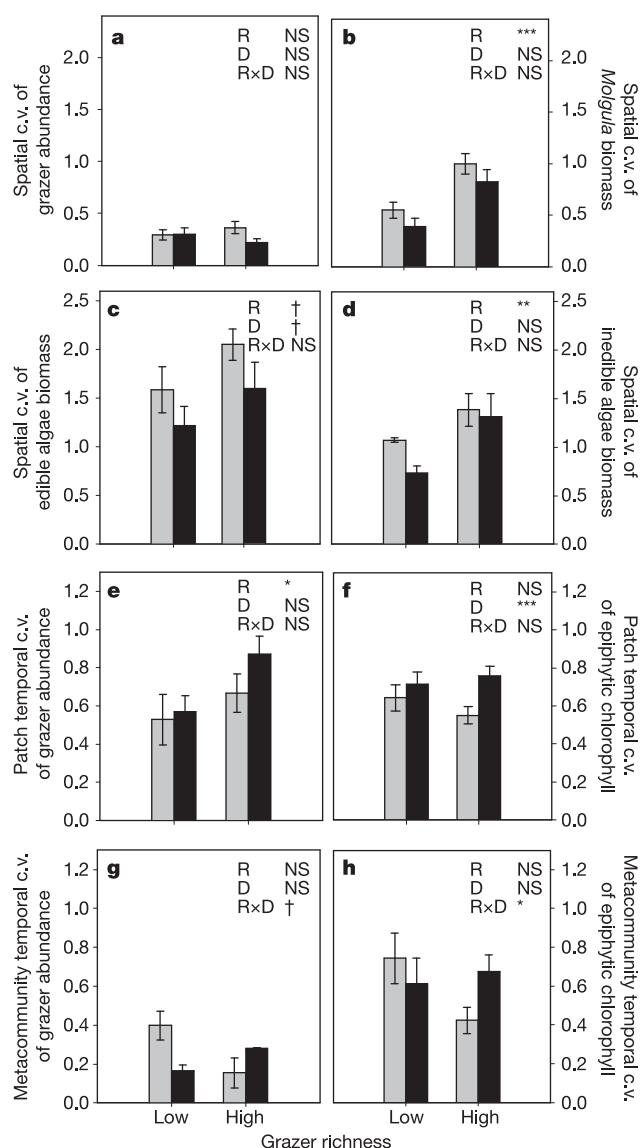


Figure 3 | Diversity effects on ecosystem variability are modified by dispersal and spatial scale. **a–d**, Spatial variation (c.v.) ($\pm$ s.e.m.) among the five patches within a metacommunity for grazer abundance (**a**), biomass of *Molgula* (the most frequent sessile invertebrate invader) (**b**), edible algae biomass (macroalgae) (**c**), and inedible algae biomass (cyanobacteria) (**d**). For each bar, $n = 5$. **e–h**, Temporal variation (c.v.) ($\pm$ s.e.m.) of grazer abundance (**e**, **g**) ($n = 10$) and epiphyte load (**f**, **h**) ($n = 25$) within patches and within whole metacommunities. Symbols are as in Fig. 1.

contradicting predictions that diverse competitive assemblages will have lower temporal variability of aggregate properties^{2,8–10,15}. Our results are consistent, however, with recent theory predicting that biodiversity can reduce stability of biomass in multitrophic food webs with strongly generalist grazers, like ours¹⁹, and that mobile consumers can destabilize production in patchy landscapes²⁵.

In contrast to patch-scale patterns, at the metacommunity scale diversity stabilized both grazer and epiphyte abundance, at least in the absence of dispersal (Fig. 3g, h), confirming predictions^{2,8–10,15}. As the patches in these unconnected metacommunities were isolated, the reduced variability of their summed properties at high diversity—even while individual patch variability was increased—must be due to asynchronous fluctuations. Asynchrony is often invoked as a mechanism stabilizing aggregate properties within patches at high diversity⁸. Similarly, spatial variability of species composition, or beta-diversity, may create asynchrony of ecosystem properties among patches, stabilizing ecosystem properties at the metacommunity scale (see the conceptual diagram, Supplementary Fig. 1). Dispersal may decrease beta-diversity and spatial heterogeneity (Fig. 1b, Fig. 3b–d) and increase synchrony, potentially eliminating this stabilizing effect (Supplementary Fig. 1). In our experiment, grazer diversity did reduce synchrony of epiphyte abundance among patches, but only without dispersal (data not shown; see Supplementary Methods). This conceptually supports the spatial insurance hypothesis for metacommunities¹⁵, but also demonstrates that diversity can contribute insurance through spatial variation even in the absence of dispersal.

The contrast between predicted stabilizing effects of diversity and dispersal, and our findings that diversity more often made ecosystem properties less predictable in space and time, highlights a potentially fundamental difference in processes mediating BD–EF relationships in single versus multitrophic ecosystems: the influence of active food and habitat selection by mobile consumers. At the metacommunity level, grazer dispersal eliminated the stabilizing effect of diversity on ecosystem properties (Fig. 3g, h), and at the patch level, grazer dispersal consistently increased temporal variability (Fig. 3e, f). Both results contradict the spatial insurance hypothesis, which is based on equilibrium metacommunities of sessile organisms with passive dispersal¹⁵. In communities of mobile animals, where dispersal is active and competitive exclusion is rare, connecting patches may allow both rapid recruitment to an optimal habitat and emigration after resource depletion, inflating temporal variability within a given patch and enhancing spatial heterogeneity. This hypothesis is also consistent with our finding that, at least in less diverse communities, dispersal enhanced grazer impacts on edible algae but reduced impacts on inedible algae (Fig. 2c, e). Habitat selection, then, might be a means by which species interactions, including those that mediate production, transcend the local scale and affect patterns at metacommunity scales^{25,26}.

Biodiversity-stability theory, like most ecological theory, assumes equilibrium^{2,8–10,15}. Although our communities experienced colonization, extinction and reached carrying capacity, they probably did not reach compositional equilibrium. Therefore, the increased temporal variability we observed in response to both diversity and dispersal might be due to transient dynamics. In nature, however, grazer composition shifts, and seagrass patches change in size and location over timescales comparable to the length of our experiment, owing to seasonal dynamics and disturbances (K.E.F. and J.E.D., unpublished observation). Because such non-equilibrium phenomena are important in most natural ecosystems, we believe our results are broadly relevant. Furthermore, running the experiment for longer would probably increase the importance of dispersal, which eliminated the predicted and observed stabilizing effects of diversity (Fig. 3e–h). Consequently, our results probably differed from diversity-stability predictions because we used mobile consumers that can actively choose patches and affect spatial heterogeneity of resources, rather than sessile organisms with passive dispersal.

Our experiment demonstrates that the stabilizing effect of biodiversity can be modified by both dispersal and scale, supporting previous theory and empirical research demonstrating that both dispersal and scale can modify the effect of biodiversity on the magnitude of productivity^{14–17}. Furthermore, our results indicate that increasing diversity will not necessarily increase the predictability of ecosystem functioning in space as it often does in time. There may be tradeoffs, then, between maximizing diversity across landscapes and stabilizing ecosystem services in time. However, our experiment also corroborates—for the first time—theory predicting that diversity can enhance reliability of ecosystem services through a spatial mechanism¹⁵: spatial heterogeneity created by more diverse metacommunities of grazers stabilized ecosystem properties at the metacommunity scale. Clearly, the spatial and temporal processes that influence diversity within natural landscapes can substantially influence the ways that biodiversity mediates ecosystem functioning. Integrating these influences is critical to effective management of ecosystem services in response to habitat fragmentation and other drivers of biodiversity change.

METHODS

Experimental organisms. *Z. marina* (eelgrass) is the most widespread and abundant marine macrophyte in the Northern Hemisphere, and it supports many commercially important species²⁷. The dominant primary consumers in many eelgrass beds are small crustacean grazers, which feed on epiphytic algae and can have important indirect, positive effects on eelgrass²⁷. We manipulated diversity of these grazers, which all have overlapping generations, direct development and summer generation times of 3–4 weeks.

Mesocosm system. The experiment was conducted in outdoor, flow-through 13.5-litre eelgrass mesocosms. Filtered seawater from the York River estuary, Virginia, USA, was delivered in pulses to mesocosms shaded to approximate natural light levels. Fifteen pre-weighed *Z. marina* shoots were planted in each mesocosm. Filters excluded grazers, but allowed passage of propagules of other invertebrates and algae^{28,29}. We grouped mesocosms into metacommunities consisting of five patches (individual mesocosms) each. For half of the 20 metacommunities, patches within the metacommunity were connected to a central hub via clear vinyl tubing with a 2.2-cm internal diameter. These dispersal corridors were 5 cm long, so grazers had an equal chance of dispersing to all other patches within the metacommunity. All grazer species could swim rapidly through the dispersal corridors, but dispersal between patches was relatively infrequent, and species differed in their dispersal inclinations (Supplementary Table S1). There was no active dispersal between unconnected patches, but these patches were linked into a metacommunity by sharing a common water supply, which was the source of propagules of all species other than the manipulated grazers.

Experimental design. We used a fully-crossed, two-factor analysis of variance (ANOVA) design with metacommunity richness and dispersal as the two factors. Metacommunity richness had two levels, low (three spp.) and high (eight spp.). The low-richness species pool was a subset of the high-richness species pool, consisting of the three most abundant grazers in the field at the time of the experiment (Supplementary Table S4). The high-richness pool included approximately 75% of the crustacean grazer species known from the lower Chesapeake Bay region (Supplementary Table S4). Each treatment combination (metacommunity richness $\times$ dispersal) was replicated five times. The initial grazer community inoculated into each mesocosm was 15 reproductively mature male–female pairs, the species composition of which was determined by random draws of pairs of individuals from the designated species pool (Supplementary Table S4). Each metacommunity initially contained the full complement of species from the whole pool, but most individual mesocosms did not. The experiment ran for 47 days, long enough for at least two complete generations of most species in addition to the founding generation, population increases approaching two orders of magnitude, and achievement of carrying capacity^{29,30}.

Sampling ecosystem properties. At two-week intervals, we estimated the biomass of epiphytic algae as epiphytic chlorophyll²⁸. Mid-way through the experiment (day 26), we sampled grazers by sweeping a dip net at mid-depth ten times, and counting and identifying the grazers captured. We estimated spatial variability of ecosystem properties as the coefficient of variation (c.v.) of each response variable across the five patches in a metacommunity. Spatial variability was initially zero, and resulted from a combination of random variation in colonization through the flow-through system and subsequent interactions with the grazer community. We also estimated temporal c.v. of epiphytic

chlorophyll (three time points) and grazer abundance (two time points). At the end of the experiment, all organisms retained by a 0.5-mm mesh sieve were separated, identified, dried to constant mass, ashed at 450 °C, and massed again.

Statistical analyses. To determine whether dispersal erased the signature of initial composition, we analysed the relationship between initial and final relative abundance for each species using a General Linear Model (GLM) with dispersal as a class predictor (Supplementary Table S1). For response variables measured within patches, we analysed data using a GLM with three factors: grazer metacommunity richness and dispersal, which were fully crossed, and metacommunity identification number (ID), which was nested within the fully-crossed design. When the *P*-value for metacommunity ID was >0.25 , we ignored that factor and ran a fully-crossed, two-way ANOVA with metacommunity richness and dispersal ($n = 25$). For response variables determined at the metacommunity level, data were analysed using a fully factorial, two-way ANOVA with metacommunity richness and dispersal as the factors ($n = 5$). We also analysed the relationship between final grazer diversity and ecosystem properties using a GLM with final Shannon–Weaver (S–W) diversity of grazers as a continuous predictor and dispersal as a class predictor. Patch S–W was used for responses in patches, and metacommunity S–W was used for metacommunity responses and spatial heterogeneity.

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LETTERS

Activity-dependent dynamics and sequestration of proteasomes in dendritic spines

Baris Bingol¹ & Erin M. Schuman¹

The regulated degradation of proteins by the ubiquitin proteasome pathway is emerging as an important modulator of synaptic function and plasticity^{1–15}. The proteasome is a large, multi-subunit cellular machine that recognizes, unfolds and degrades target polyubiquitinated proteins. Here we report NMDA (*N*-methyl-D-aspartate) receptor-dependent redistribution of proteasomes from dendritic shafts to synaptic spines upon synaptic stimulation, providing a mechanism for local protein degradation. Using a proteasome-activity reporter and local perfusion, we show that synaptic stimulation regulates proteasome activity locally in the dendrites. We used restricted photobleaching of individual spines and dendritic shafts to reveal the dynamics that underlie proteasome sequestration, and show that activity modestly enhances the entry rate of proteasomes into spines while dramatically reducing their exit rate. Proteasome sequestration is persistent, reflecting an association with the actin-based cytoskeleton. Together, our data indicate that synaptic activity can promote the recruitment and sequestration of proteasomes to locally remodel the protein composition of synapses.

To examine whether synaptic activity regulates proteasome localization at synapses, we expressed the 19S proteasome subunit Rpt1 fused to green fluorescent protein (GFP) in cultured hippocampal neurons¹⁶. Biochemical analyses showed that the Rpt1–GFP subunit was incorporated into the endogenous proteasome complex at

~77% of the efficiency of another endogenous proteasome subunit (Supplementary Fig. S1a). Under control conditions, Rpt1–GFP was diffusely distributed in both dendrites and spines (Fig. 1a). After depolarization (60 mM KCl, 1.5 min), Rpt1–GFP moved from dendritic shafts into spines within minutes (Fig. 1a–c). Although there was no significant change in the total amount of Rpt1–GFP fluorescence after KCl stimulation, there was on average a ~90% increase in the spine Rpt1–GFP signal (20 min after KCl) (Fig. 1c, d). The increase in spine proteasome signal was persistent, typically lasting for at least 1 h. Most of the Rpt1-occupied spines showed overlap with a presynaptic protein, bassoon (Supplementary Fig. S3a and Supplementary Table S1), indicating that these spines are the sites of functional synapses.

The depolarization-induced redistribution of proteasomes does not reflect bulk movement of proteins into spines (Supplementary Fig. S1b–e), nor can it be explained by an increase in the number or area of spines (Supplementary Figs S1b–g and S2). However, redistribution into spines was also observed with a fluorescently tagged 20S proteasome subunit, $\alpha 4$, indicating that both 19S and 20S proteasome subunits undergo activity-dependent trafficking (Supplementary Fig. S1f, g).

Depolarization with KCl leads to the activation of many voltage-gated channels, including NMDA-type glutamate receptor channels, which are critical for the initiation of synaptic plasticity in the

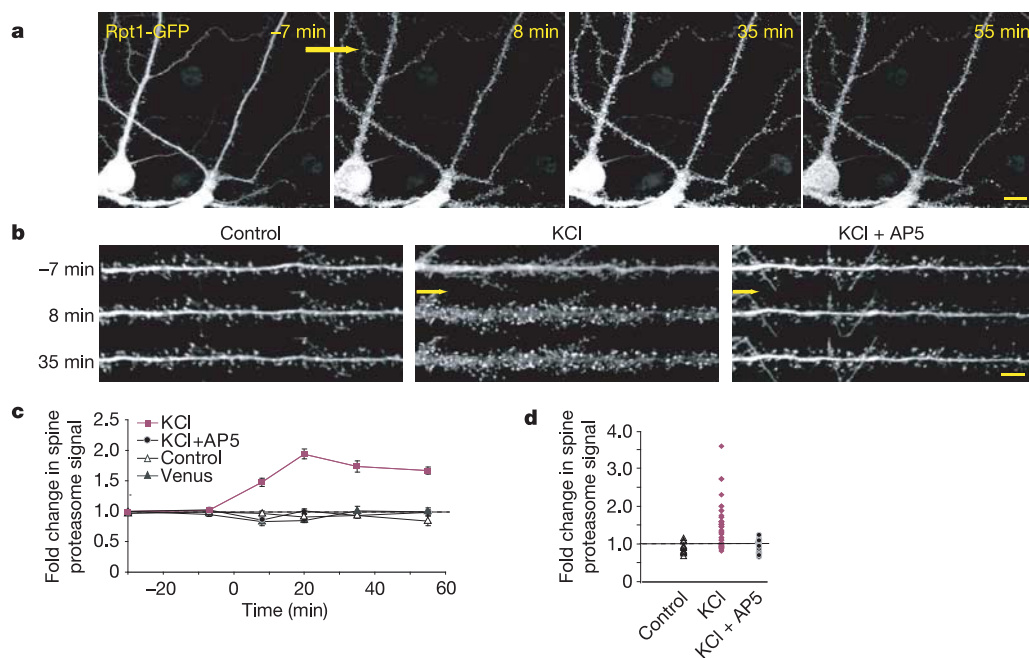


Figure 1 | A GFP-labelled proteasome subunit, Rpt1-GFP, moves into spines upon depolarization. **a**, Time-lapse imaging of Rpt1–GFP-labelled neurons. Arrow indicates time of stimulation with KCl. Scale bar, 11 μ m. **b**, Higher-resolution images of dendrites under the indicated conditions. Scale bar, 6 μ m. **c**, Summary data showing quantification of mean spine signal intensity (from top to bottom, $n = 37, 21, 11$ and 21 cells from 7 independent experiments). The KCl group differs significantly from all others starting at $t = 8$ min ($P < 0.01$). **d**, Change in signal level in spines (at $t = 35$ min) for each individual experiment. Error bars indicate s.e.m.

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central nervous system¹⁷. The NMDA receptor antagonist AP5 (D(-)-2-amino-5-phosphonovaleric acid) blocked KCl-induced movement of both Rpt1-GFP (Fig. 1c, d) and $\alpha 4$ -venus fusion proteins (Supplementary Fig. S1g), indicating that NMDA receptor activity is required. We also observed that direct stimulation with NMDA (20 μ M, 3 min) was sufficient to drive the redistribution of Rpt1-GFP into spines (mean per cent increase in spine signal $35.6 \pm 2.8\%$; $n = 10$ cells per group; $P < 0.05$). Together, these data suggest that NMDA receptor activation specifically recruits proteasomes from dendritic shafts into spines.

We next examined whether endogenous proteasomes show a similar activity-dependent redistribution. First, we observed that most Rpt1-GFP-labelled spines also showed positive immunolabelling for core (20S) endogenous proteasome subunits (Supplementary Fig. S3b and Supplementary Table S1) and actinin (data not shown). We also observed that stimulation (60 mM KCl, 1.5 min) caused a significant increase in the mean intensity of endogenous synaptic proteasome particles, without changing their area or number (Fig. 2a, b). However, the total intensity of the proteasome signal, including all shafts and spines, did not change. Synaptosomes prepared from stimulated hippocampal slices (60 mM KCl, 6 min) revealed a specific increase in the level of synaptic proteasomes, whereas other synaptic proteins showed no change (Fig. 2c, d). Thus, endogenous proteasomes move into synapses during stimulation in both cultured hippocampal neurons and hippocampal slices.

Is proteasome recruitment accompanied by a change in the level of ubiquitinated synaptic proteins? To investigate this, we used an antibody that recognizes poly- and monoubiquitinated proteins

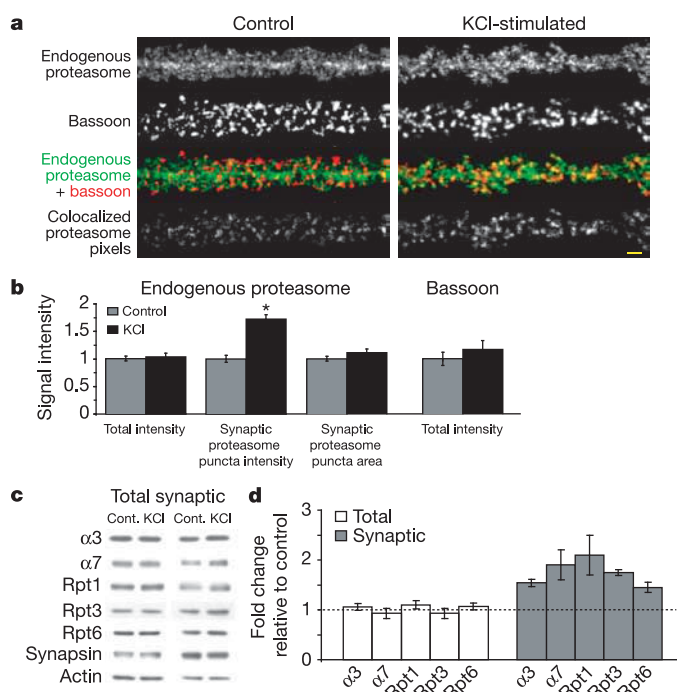


Figure 2 | Endogenous proteasomes move into spines. **a**, Stimulation with KCl also increases endogenous synaptic proteasomes. Immunostaining was performed (12 min after stimulation) using two different antibodies against proteasome components and a presynaptic marker (against bassoon). Scale bar, 3 μ m. **b**, Analysis of endogenous proteasomes (control, $n = 17$ cells; KCl, $n = 24$ cells from 3 independent experiments; $P < 0.05$ for the mean particle intensity), with mean intensity and area of individual proteasome puncta shown. **c**, Western blot analysis of proteasome subunits in total lysate and synaptic fractions. Enrichment of endogenous proteasomes can be detected in synaptosomes prepared from KCl-stimulated slices. Synapsin and actin were used as controls. **d**, Group data for the slice experiment shown in **c** ($n = 3$ experiments; $P < 0.05$). Error bars indicate s.e.m.

but not free ubiquitin (clone FK2)^{2,18}. Depolarization caused an initial $\sim 67\%$ increase in ubiquitin levels in the spine and shaft 10 min after stimulation (Supplementary Fig. S4a, b). At later time points, there was a proteasome-inhibitor-sensitive decrease in ubiquitinated protein levels in spines. Note that the time at which the maximum increase in spine proteasome signal was observed (Fig. 1c) coincides with the time at which we observed the decrease in the ubiquitinated proteins (Supplementary Fig. S4a, b).

We also examined whether stimulation with KCl can locally regulate proteasome activity using a well-characterized, GFP-based proteasomal degradation reporter that harbors a ubiquitin degradation signal (Ub^{G76V}-GFP; refs 19, 20) and loses fluorescence upon degradation. Stimulation of neurons (60 mM KCl, 1.5 min) caused a

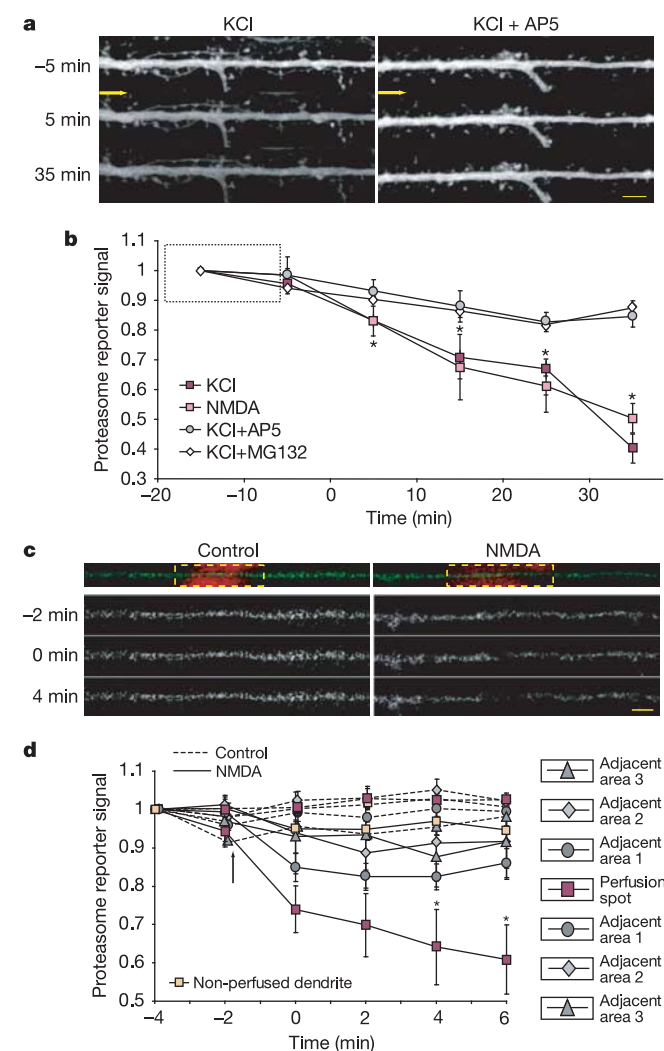


Figure 3 | KCl stimulation increases proteasome activity. **a**, Time-lapse images of dendrites from neurons expressing Ub^{G76V}-GFP. Bath application of KCl (arrow) resulted in an NMDA-receptor-dependent loss of fluorescence, reflecting reporter degradation. Scale bar, 7.5 μ m. **b**, Addition of KCl or NMDA caused a significant drop in fluorescence that was prevented by AP5 or proteasome inhibitor MG132 ($n = 8$ cells per group, 4 independent experiments; $P < 0.05$ at $t = 5$ min and thereafter). The dashed box refers to the region of interest in Supplementary Fig. S5b, c. **c**, Time-lapse images of dendrites from neurons expressing Ub^{G76V}-GFP, locally perfused with NMDA or control solution. The position of the local perfusion is shown in red (yellow box). Perfusion starts at 0 time point. See also Supplementary Fig. S5d, e. Scale bar, 3.5 μ m. **d**, Change in reporter signal at the perfused, adjacent and non-perfused dendrites ($n = 4$ cells per group, 3 independent experiments; $P < 0.05$ at $t = 4$ min and thereafter). Error bars indicate s.e.m.

rapid proteasome-inhibitor-sensitive (Supplementary Fig. S5a) and NMDA-receptor-dependent decrease in the reporter signal, indicating proteasome activity during depolarization (Fig. 3a, b). Consistent with this, direct activation of NMDA receptors (20 μ M NMDA, 3 min) was sufficient to decrease the reporter signal (Fig. 3b). Closer inspection indicated a more rapid loss of fluorescence in the spines (within ~ 2 min) than in the shaft (Supplementary Fig. S5b, c). This was followed by apparently equal rates of reporter degradation in the spines and shaft, possibly owing to diffusion of the reporter from the shaft to the spine (where it would be degraded). To examine whether proteasomes can be activated locally, we perfused NMDA locally and monitored reporter fluorescence (Supplementary Fig. S5d, e). There was a spatially restricted decrease in the intensity of the reporter signal at the perfused spot ($\sim 60\%$ decrease), whereas adjacent areas showed only very modest reductions ($\sim 15\%$) (Fig. 3c, d) and non-perfused dendrites showed only a 5% decrease (Fig. 3d). In control (no NMDA) perfusions, there was no change in the GFP signal (Fig. 3c, d). These results show that local protein degradation can occur in dendrites and can be activated by NMDA receptor activity.

To understand the dynamics underlying proteasome enrichment in the spines, we monitored Rpt1-GFP fluorescence recovery after photobleaching (FRAP). In the absence of stimulation, individual spines showed ~ 50 – 65% recovery of the spine Rpt1 signal after a single or multiple bleaching episodes (Fig. 4a, c). To examine how synaptic stimulation alters the dynamics of proteasome localization, we monitored FRAP in the same individual spines before and after stimulation with KCl. Before stimulation, spines showed about 65% fluorescence recovery (Fig. 4b, c). After stimulation with KCl, which

resulted in an increase in the spine Rpt1 signal (Fig. 4b), there was a dramatic decrease in fluorescence recovery, down to $\sim 10\%$ (Fig. 4b, c). Control, unbleached spines showed minimal fluorescence during the FRAP experiment. The decreased recovery of Rpt1-GFP in spines can not be explained by a decrease in the shaft source fluorescence as decreasing the shaft source fluorescence deliberately (by bleaching) to a reduced level comparable to that observed following stimulation had no effect on the extent of Rpt1-GFP fluorescence recovery (Fig. 4d). We thus conclude that the immobile fraction of spine proteasomes increases from 35% to 90% upon stimulation, indicating that the proteasome is actively sequestered in spines.

We reasoned that sequestration of proteasomes in spines may be due to a decrease in the rate of proteasome exit from the spines. To test this, we performed fluorescence loss in photobleaching (FLIP) experiments by repeatedly bleaching an area of the dendritic shaft and monitoring the loss of Rpt1 signal in the adjacent spine (and a control spine) after each bleaching episode. Before KCl stimulation, repeated bleaching of the shaft was associated with a dramatic loss of Rpt1 spine fluorescence owing to movement of fluorescently tagged proteasomes out of spines and into the shafts, where they subsequently underwent bleaching (Fig. 4e, f). After stimulation, there was an increase in the Rpt1-GFP signal in spines that was much more resistant to repeated bleaching of the shaft (Fig. 4e, f). Analysis showed that stimulation caused an increase in the immobile fraction of Rpt1 in the spines (from $\sim 16\%$ to $\sim 90\%$). Together, our FRAP and FLIP experiments reveal that stimulation with KCl enhances the proteasome entry rate by ~ 1.5 -fold and decreases the exit rate by at

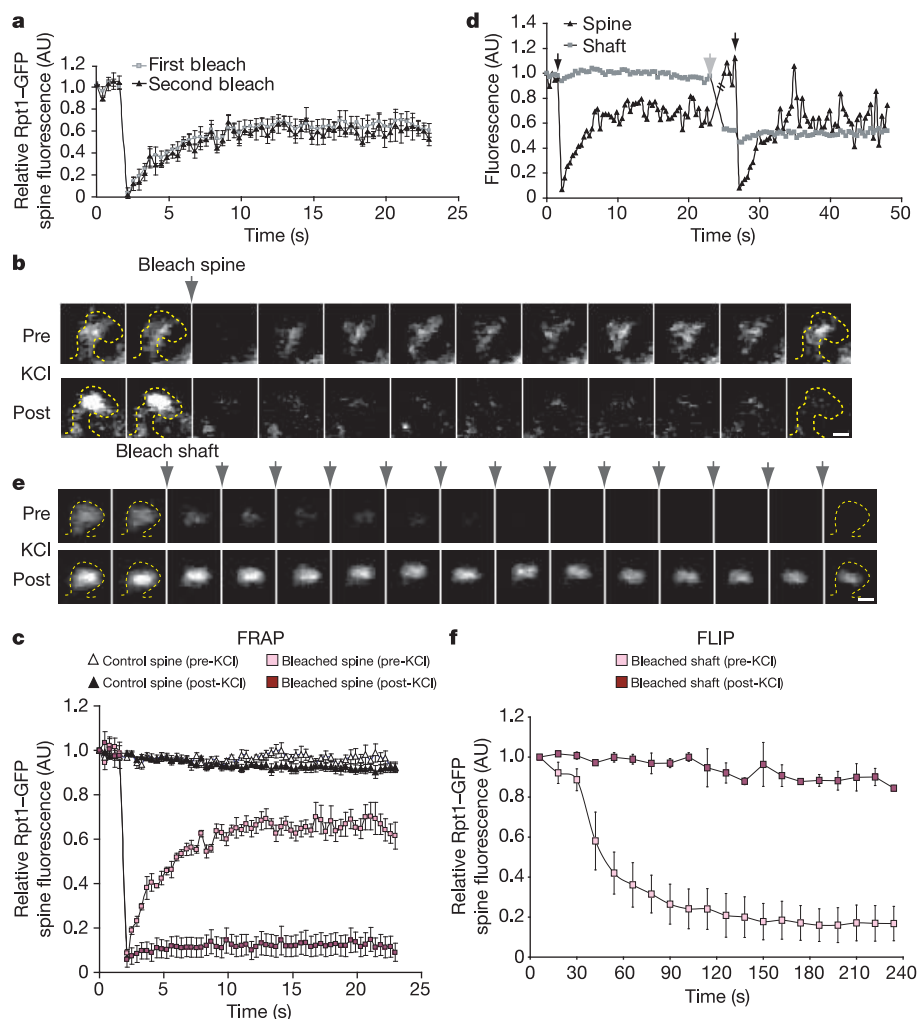


Figure 4 | Photobleaching of Rpt1-GFP indicates the tight association of proteasomes with spines.

a, In the absence of KCl stimulation, FRAP in spines is similar with repeated bleaching ($n = 9$ cells from 4 independent experiments). Fluorescence levels are shown in arbitrary units (AU). **b**, In a representative FRAP experiment, a single spine was bleached (arrow) before (pre-) and after (post-) KCl stimulation. Fluorescence recovery is greatly reduced after stimulation. Scale bar, 0.75 μ m. **c**, Bleaching of spines was followed by significant recovery of fluorescence before KCl stimulation ($\tau = 2.9 \pm 2.0$ s), but recovery was significantly reduced after stimulation ($\tau = 2.0 \pm 0.5$ s; $n = 9$ cells from 4 experiments; $P < 0.01$). (τ , time constant). The fluorescence signal in each group is normalized to itself at $t = 0$. **d**, FRAP, monitored in a single spine, was equivalent before and after bleaching (to 50%) of the dendritic shaft ($n = 3$). Parallel diagonal lines on the curve indicate the start of the second FRAP. **e**, Representative FLIP experiment in which Rpt1 fluorescence in a single spine is monitored after repeated bleaching (arrows) of the associated dendritic shaft, before and after KCl stimulation. With KCl stimulation (note the enhanced Rpt1 signal in the spine), there is much less fluorescence loss in the spine after shaft bleaching, indicating reduced mobility of Rpt1. Scale bar, 0.75 μ m. **f**, Analysis of FLIP experiments ($n = 4$ cells from 3 independent experiments; $P < 0.01$). Each group is normalized to itself at $t = 0$. (FLIP pre-stimulation τ , 38.3 ± 2.4 s; post-stimulation, the data were better fit by a linear function.) Error bars indicate s.e.m.

least sixfold. We suggest that the proteasome sequestration induced by stimulation is largely due to a decreased exit rate from spines (possibly due to enhanced protein–protein interactions), and to a much lesser extent due to the increased entry rate.

How do proteasomes become concentrated in spines? To study the potential association of proteasomes with the cytoskeleton, we performed detergent extraction experiments²¹. (Proteins resistant to detergent extraction are more tightly associated with the cytoskeleton.) A large proportion of the proteasomes could not be extracted with detergent: ~70–80% of proteasome labelling remained after detergent extraction compared to non-extracted cultures (Fig. 5a, b). In fact, proteasome subunits more closely resembled the cytoskeleton-associated protein α -actinin-2 than other synaptic proteins²¹ that are less tightly associated with the cytoskeleton (Fig. 5a, b). The partial extractability of the proteasome was also confirmed by western blot analysis (data not shown). To estimate the fraction of proteasomes associated with the actin cytoskeleton in the Triton-resistant population, we incubated neurons with the actin-disrupting agent latrunculin A before detergent extraction²¹. Latrunculin A treatment increased the detergent extractability of proteasomes to a similar extent as α -actinin (Fig. 5a, b), suggesting that the fraction of proteasomes targeted by detergent extraction is mostly associated with the actin cytoskeleton. On the basis of these data, we estimate that ~50% of the proteasomes in dendrites are associated with the actin cytoskeleton. We confirmed this partial association by immunolabelling for proteasome subunits and actin (Supplementary Fig. S3c and Supplementary Table S1). Furthermore, after treatment with latrunculin A alone, we observed that most of the proteasome puncta disappeared (Fig. 5a, b). Latrunculin A treatment affected α -actinin in a similar manner, but did not affect

synaptophysin or calcium/calmodulin-dependent protein kinase II (CaMKII) greatly (Fig. 5a, b). Together, these data suggest that a substantial fraction of the proteasomes in hippocampal neurons is associated with the actin cytoskeleton.

To test whether activity regulates the association of proteasomes with the actin cytoskeleton, we stimulated hippocampal cultures (20 μ M NMDA, 1 min), detergent-extracted and then immunostained for proteasome subunits and actin. NMDA stimulation significantly increased the number of actin-associated proteasome subunits (Fig. 5c, d). Immunoblotting of the detergent-resistant proteasome population also revealed a significant increase in the level of proteasomes that were not extracted (that is cytoskeleton-associated) compared to the unstimulated controls (Fig. 5e, f). These data show that stimulation with NMDA leads to an increase in proteasome association with the actin cytoskeleton, suggesting a plausible mechanism for its sequestration after stimulation. In contrast, there seems to be little involvement of the microtubule-based cytoskeleton (Supplementary Fig. S6a, b)^{22,23}.

Recent studies of ubiquitin-proteasome system (UPS) regulation have emphasized the importance of substrate recognition and ubiquitination by the enzymes of the conjugation pathway²⁴. Downstream of ubiquitination, E3 and other proteins²⁵ have been proposed to 'deliver' the target proteins to the proteasome. The converse possibility, involving movement of a proteasome to the target protein, is an emerging concept²⁶, with much less direct experimental support. It is worth noting, however, that biochemical studies have shown an apparent interaction of proteasomes with proteins physically proximal to degradation targets²⁶. In addition, movement of proteasomes between the cytoplasm and nucleus has been observed during mitosis and meiosis²⁷.

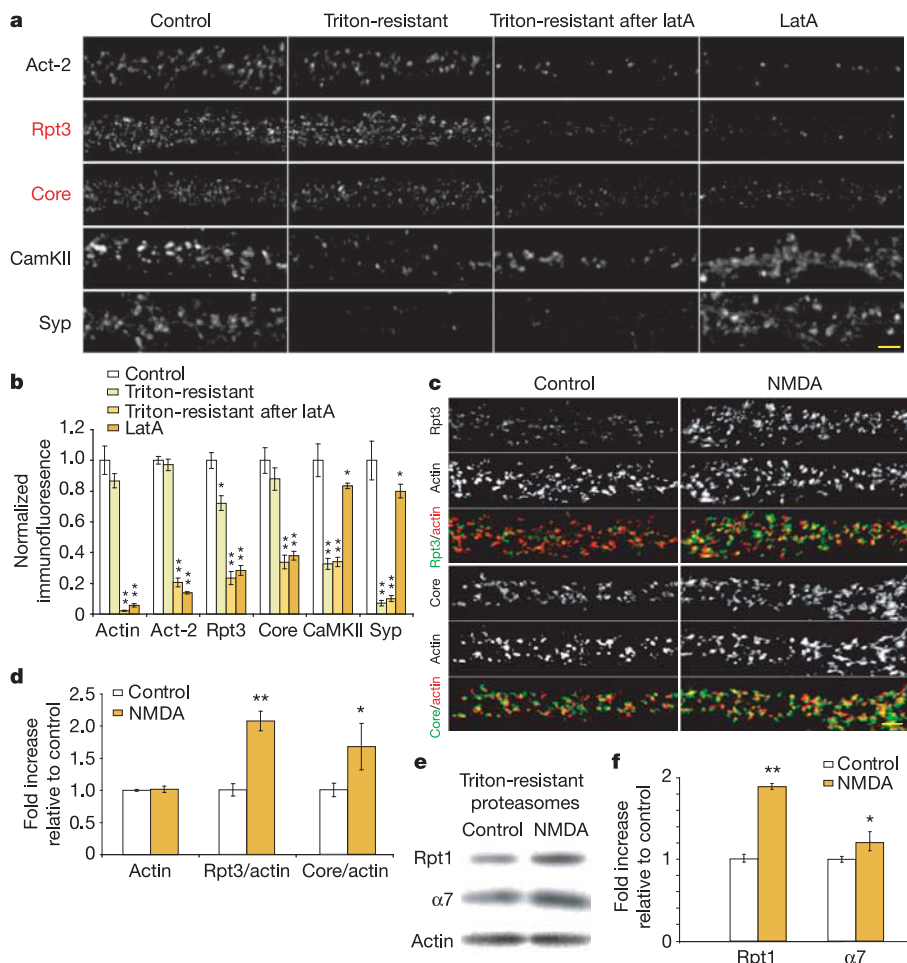


Figure 5 | The proteasome associates with the actin cytoskeleton. **a**, Immunostaining for proteasome subunits, α -actinin-2 (Act-2), CaMKII and synaptophysin (Syn) in control neurons and neurons exposed to Triton X-100 or latrunculin A alone or sequentially. Scale bar, 4 μ m. **b**, Group data for **a** ($n = 25$ cells per group, 3 independent experiments). Asterisk, $P < 0.05$; two asterisks, $P < 0.01$. **c**, Stimulation with NMDA increases the number of actin-bound proteasome subunits. Only proteasome puncta that are associated with an actin puncta are shown. Scale bar, 4 μ m. **d**, Proteasome colocalization values were normalized to the actin staining within each dendrite ($n = 40$ from 3 independent experiments; $P < 0.01$ for Rpt3 and $P < 0.05$ for core subunits). **e**, **f**, Stimulation with NMDA increases the pool of proteasomes associated with the actin cytoskeleton ($n = 4$ independent experiments, $P < 0.01$ for Rpt1 and $P < 0.05$ for α 7). Error bars denote s.e.m.

The activity-dependent dynamics described here suggest that proteasomes can be recruited to locally sculpt the protein composition of the synapse, providing on-site degradation rather than serving as something akin to a remote garbage-disposal site. In support of this idea, others have observed the association of proteasomes with the cytoplasmic tails of receptor complexes²⁸, suggesting a capacity for rapid remodelling of membrane-associated receptor complexes^{3,4,7–15}. Here we show dynamic, NMDA receptor-dependent redistribution of proteasomes from dendritic shafts to spines. Together with the detection of more polyribosomes in potentiated spines²⁹, our data suggest the capacity for local remodelling of spines through the coordinated synthesis and degradation of proteins^{13–15}. In our experiments, the long-lasting nature of proteasome recruitment to the spines indicates the possibility of local and persistent remodelling that outlasts the stimulation period.

METHODS

See Supplementary Information for detailed methods.

Viral constructs. Rpt1–GFP was cloned into the pSinRep5 vector (Invitrogen) from the pBS-Rpt1–GFP–HU plasmid¹⁶. Rpt1–GFP–IRES–mRFP was generated from the pSinRep5–Rpt1–GFP clone by inserting IRES and mRFP sequences downstream of the Rpt1–GFP sequence. To generate the α 4–venus Sindbis virus, the α 4 cDNA (MGC accession 2581897) was cloned upstream of venus sequence (pCS2–venus; ref. 30) in pSinRep5. The degradation reporter Ub^{G76V}–GFP construct (refs 19, 20) was cloned into pSinRep5 from the EGFP–N1–Ub^{G76V}–GFP vector.

Cultured hippocampal neurons and live imaging. Dissociated postnatal (P)1–2 rat hippocampal neuronal cultures (18–21 days *in vitro*) were used in all experiments. Images were acquired on a Zeiss LSM 510 microscope (X40 oil-immersion objective) for tagged proteasome experiments and on an Olympus IX50 microscope (X40 objective) for proteasome activity experiments.

Statistical analysis. Unpaired, two-tailed Student's *t*-tests and one-way analysis of variance (ANOVA) were used for statistical analyses. All data were tested for normal distribution.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition

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Legumes, such as *Medicago truncatula*, form mutualistic symbiotic relationships with nitrogen-fixing rhizobial bacteria. This occurs within specialized root organs—nodules—that provide the conditions required for nitrogen fixation. A rhizobium-derived signalling molecule, Nod factor, is required to establish the symbiosis. Perception of Nod factor in the plant leads to the induction of Ca^{2+} oscillations¹, and the transduction of this Ca^{2+} signal requires *DMI3* (refs 2, 3), which encodes the protein kinase Ca^{2+} /calmodulin-dependent protein kinase (CCaMK). Central to the regulation of CCaMK is an autoinhibitory domain that negatively regulates kinase activity. Here we show that the specific removal of the autoinhibition domain leads to the autoactivation of the nodulation signalling pathway in the plant, with the resultant induction of nodules and nodulation gene expression in the absence of bacterial elicitation. This autoactivation requires nodulation-specific transcriptional regulators in the GRAS family. This work demonstrates that the release of autoinhibition from CCaMK after calmodulin binding is a central switch that is sufficient to activate nodule morphogenesis. The fact that a single regulation event is sufficient to induce nodulation highlights the possibility of transferring this process to non-legumes.

CCaMK can bind calcium in two forms: free Ca^{2+} ions, which bind directly to three EF-hand domains, and Ca^{2+} ions complexed with calmodulin (CaM)^{4,5}. The binding of free Ca^{2+} ions induces autophosphorylation and this enhances the binding of CaM, which in turn promotes substrate phosphorylation⁶. Here we show that CCaMK from *M. truncatula* (*DMI3*) has a similar two-step regulation by Ca^{2+} ions: Ca^{2+} -induced autophosphorylation of *DMI3* (Fig. 1b) with a specific activity of 15.3 pM ^{32}P incorporated min^{-1} per mg of protein in 0.5 mM CaCl_2 , and Ca^{2+} -CaM binding (Fig. 1a) activated substrate phosphorylation (Fig. 1c) with a higher specific activity (10.1 nM ^{32}P incorporated min^{-1} per mg of protein in 0.5 mM CaCl_2 and 0.5 μM CaM). Direct binding of Ca^{2+} ions is essential for CCaMK activity, because the removal of any of the EF-hand domains abolishes complementation of the *dmi3-1* mutation (Fig. 2a), even though the proteins are stable and appropriately localized (Supplementary Fig. S1).

A significant proportion of the previous biochemical work on CCaMK has been performed on the protein from *Lilium longiflorum* that shows 72% identity with *M. truncatula* *DMI3* (refs 2–4). We presume that CCaMKs in non-legumes such as *L. longiflorum* function in the establishment of the mycorrhizal fungal symbiosis, which is also a function of *DMI3* in *M. truncatula*. We have shown that *DMI3* has equivalent biochemical activity *in vitro* to that of *L. longiflorum* CCaMK. Further support of this is the fact that *L. longiflorum* CCaMK can complement the *dmi3-1* mutant for the generation of nodules (Fig. 1d). Therefore, despite the fact that *L. longiflorum* does not form nodules, the *L. longiflorum* CCaMK

protein functions in an analogous manner to *DMI3* during nodulation signalling in legumes.

An autoinhibition domain is central to the regulation of CCaMK, and this domain overlaps with a CaM-binding domain such that autoinhibition is suppressed by CaM binding^{4,7}. *DMI3* contains a predicted CaM-binding/autoinhibition domain between residues 326 and 355, and the removal of this domain abolishes CaM binding (Fig. 1a). *DMI3* deletions specifically lacking the CaM binding/autoinhibition domain (*DMI3*Δ328–355) and deletions that isolate the *DMI3* kinase from the rest of the protein (*DMI3* 1–326 and *DMI3* 1–311) lead to constitutive substrate phosphorylation *in vitro* that is independent of Ca^{2+} and CaM (Fig. 2b), although the levels of activity are less than that of the Ca^{2+} -CaM induced activity of the full-length protein. *DMI3* 1–346, which contains the kinase domain with the CaM-binding/autoinhibitory domain, shows almost no substrate phosphorylation *in vitro* (Fig. 2b), indicating that the CaM-binding/autoinhibitory domain negatively regulates *DMI3* activity.

We introduced these constitutively active constructs under the control of the 35S promoter into wild-type and *dmi3-1* lines containing the Nod-factor reporter *ENOD11-GUS*, in which *GUS* encodes β -glucuronidase. In both the wild type and the *dmi3-1* mutant, similar effects were seen with the deletion constructs. Roots transformed with

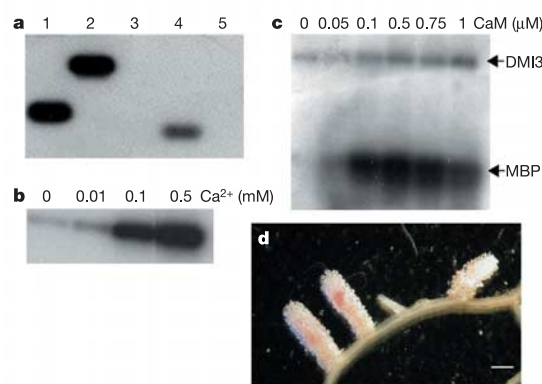


Figure 1 | *L. longiflorum* CCaMK and *M. truncatula* *DMI3* share biochemical properties. **a**, *L. longiflorum* CCaMK (lane 1), *DMI3* (lane 2) and *DMI3* 1–346 (lane 4) bind ^{35}S -labelled recombinant CaM, but *DMI3* 1–326 (lane 3) and *DMI3*Δ328–355 (lane 5), which lack the CaM-binding domain, do not. **b**, *DMI3* autophosphorylation is dependent on Ca^{2+} and is modified by the Ca^{2+} concentration. **c**, *DMI3* in the presence of CaM and 0.1 mM Ca^{2+} phosphorylates myelin basic protein (MBP) and this is dependent on the concentration of CaM. **d**, Transformation of *dmi3-1* roots with 35S CCaMK from *L. longiflorum* leads to complementation as indicated by the formation of nodules. Scale bar, 1 mm.

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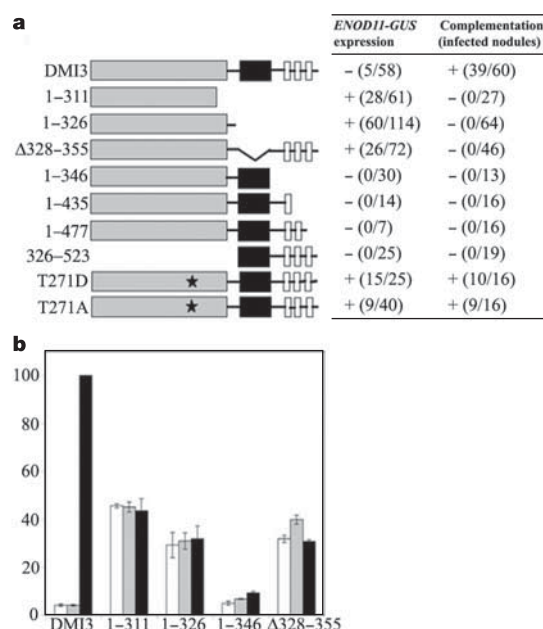


Figure 2 | The *DMI3* mutations driven by the 35S promoter generated to study kinase activity, induction of *ENOD11-GUS* and complementation.

a, *DMI3* encodes a CCaMK with a serine/threonine kinase domain (grey), an autoinhibitory domain that overlaps a CaM-binding domain (black) and three EF-hand motifs (white). The numbers in parentheses indicate the number of plants that tested positive for *ENOD11-GUS* staining in the absence of Nod factor or the number of plants with bacterially infected nodules in complementation tests with *dmi3-1*, out of the total number of plants tested. **b**, Substrate (GS peptide) phosphorylation of the deletion constructs relative to fully activated *DMI3* indicates constitutive activity of *DMI3* 1–311, *DMI3* 1–326 and *DMI3*Δ328–355. White bars, without Ca^{2+} ; grey bars, with Ca^{2+} ; black bars, with Ca^{2+} /CaM. Results are means $\pm$ s.d. Values along the y axis indicate percentage substrate phosphorylation relative to *DMI3* with Ca^{2+} /CaM.

35S-*DMI3* showed non-symbiotic GUS expression in the root cap and vascular tissues (Fig. 3a), with occasional (9% of roots) light GUS activity in epidermal cells. When 35S-*DMI3* roots were incubated in 1 nM Nod factor for 6 h, strong GUS expression occurred in epidermal cells in 64% of root systems (30 of 47 plants), with GUS activity limited to a zone of epidermal cells associated with root hair outgrowth and maturation (Fig. 3b, c), young epidermal cells lacked expression and older epidermal cells showed patchy expression, as reported previously⁸. Roots transformed with *DMI3* 1–326, *DMI3* 1–311 and *DMI3*Δ328–355 showed strong *ENOD11-GUS* expression in epidermal cells, primarily of lateral roots, in the absence of Nod factor treatment in 53%, 46% and 36% of root systems, respectively (Figs 2a and 3d–f). The *ENOD11-GUS* expression induced by these deletion constructs was observed in a similar zone of epidermal cells to that of the *ENOD11* induction by Nod factor. This indicates that much of the spatial regulation of this response is a function of components that act later than CCaMK. The induction of the native *ENOD11* gene in roots transformed with *DMI3* 1–326 was confirmed by polymerase chain reaction with reverse transcription (RT-PCR) (Fig. 3i). No induction of *ENOD11-GUS* was seen with *DMI3* 1–346, which contains the autoinhibitory domain, or with *DMI3* 326–523, which specifically lacks the kinase domain (Fig. 2). Green fluorescent protein (GFP) contained within the transfer DNA (T-DNA) was used as an indication of transformation in these experiments. Together, these data indicate that *DMI3* coordinates the expression of nodulation genes and that the regulation of the kinase through autoinhibition is central to the regulation of this protein.

To assess the activity of the gain-of-function constructs further, we

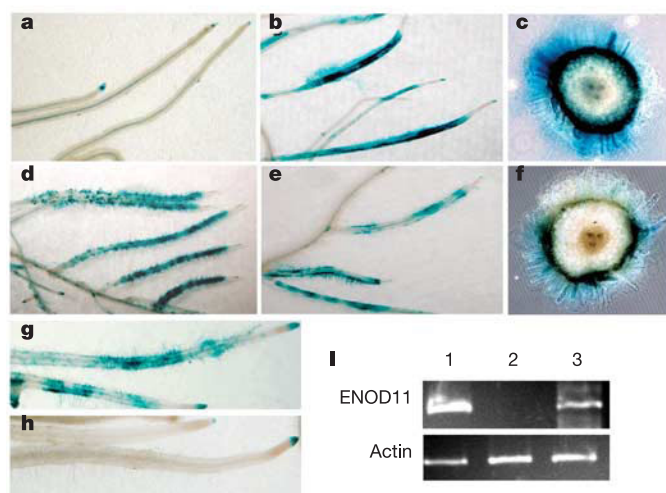


Figure 3 | Induction of *ENOD11* by *DMI3* gain-of-function constructs.

a, b, Roots of *dmi3-1* transformed with 35S-*DMI3* show little GUS activity (**a**) but show activation of *ENOD11-GUS* when treated with 1 nM Nod factor (**b**). **c**, Cross-sections show an epidermal predominance of GUS staining. **d, e**, Roots transformed with 35S-*DMI3* 1–326 (**d**) and 35S-*DMI3*Δ328–355 (**e**) show *ENOD11-GUS* activity in the absence of Nod factor. **f**, Cross-sections of 35S-*DMI3* 1–326 indicate epidermal GUS expression. **g, h**, 35S-*DMI3* 1–326 can activate *ENOD11-GUS* in *dmi2-1* mutants (**g**) but not in *nsp2-1* mutants (**h**). Original magnification: **a, b, d, e**, $\times 10$; **c, f**, $\times 25$; **g, h**, $\times 15$. **i**, RT-PCR shows that native *ENOD11* is induced in root transformed with 35S-*DMI3* 1–326. Lane 1, *dmi3-1* transformed with 35S-*DMI3* treated with 1 nM Nod factor; lane 2, *dmi3-1* transformed with 35S-*DMI3*; lane 3, *dmi3-1* roots transformed with 35S-*DMI3* 1–326.

transformed wild-type and *dmi3-1* plants with *DMI3* 1–311 and *DMI3* 1–326 expressed from the *DMI3* native promoter (*DMI3p*): both constructs activated *ENOD11-GUS*. However, in addition we observed the formation of nodules even when no Nod factor or bacteria were present (Fig. 4d, e). These spontaneous nodules occurred in sterile culture, and no nodules were ever observed under such conditions with control *dmi3-1* plants transformed with *DMI3p-DMI3*. Sectioning of these spontaneous nodules showed the absence of a bacteria-infected zone (Fig. 4d) which is seen if *dmi3-1* plants are complemented by *DMI3p-DMI3* and inoculated with the symbiotic bacterium *Sinorhizobium meliloti* (Fig. 4b). 43% of plants (20 of 47) transformed with *DMI3p-DMI3* 1–311 showed spontaneous nodules, with an average of 5.4 ± 1.3 (mean $\pm$ s.e.m.) nodules per plant, and 53% of plants (16 of 30) transformed with *DMI3p-DMI3* 1–326 showed spontaneous nodules, with an average of 4.4 ± 1.1 nodules per plant, all in the absence of *S. meliloti* and Nod factor. For comparison, after inoculation with *S. meliloti* there was an average of 17.1 ± 3.4 nodules per plant when the *dmi3-1* mutant was complemented by *DMI3p-DMI3* (Fig. 4a), and 7.2 ± 1.6 nodules per plant with 35S-*DMI3*. Spontaneous nodules induced by *DMI3p-DMI3* 1–311 showed appropriate *ENOD11-GUS* induction (Fig. 4e). When we inoculated *S. meliloti* onto roots transformed with *DMI3p-DMI3* 1–311 or *DMI3p-DMI3* 1–326 we were unable to detect bacteria inside the nodules (Figs 2a and 4c) and saw little indication of root hair responses and no development of infection threads (Fig. 4f, g). Hence, even though *DMI3p-DMI3* 1–311 and *DMI3p-DMI3* 1–326 lead to the formation of nodules, they are not sufficient to fully complement the *dmi3-1* mutation to allow bacterial entry.

Ca^{2+} -induced autophosphorylation of CCaMK enhances CaM binding and has therefore been predicted to promote substrate phosphorylation⁵. To assess the function of autophosphorylation further, we mutated the autophosphorylated threonine residue in *DMI3* to aspartate and alanine (*DMI3* T271D and *DMI3* T271A, respectively). *DMI3* T271D, and to a smaller extent *DMI3* T271A,

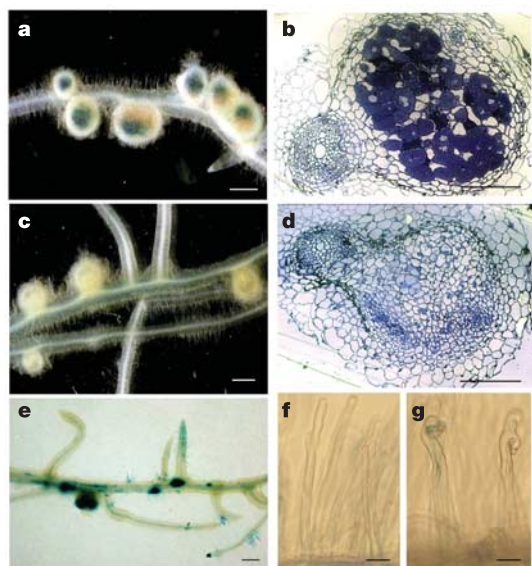


Figure 4 | Spontaneous nodulation. **a**, Nodules on *dmi3-1* complemented by DMI3p-DMI3. Bacteria in the nodules stain blue as a result of LacZ activity. Scale bar, 1 mm. **b**, A 0.5- μ m section of a nodule from *dmi3-1* complemented with DMI3p-DMI3; bacteria within the nodule are darkly stained. Scale bar, 0.25 mm. **c**, *dmi3-1* roots transformed with DMI3p-DMI3 1–311 induce spontaneous nodules that show no bacterial invasion after inoculation with *S. meliloti*. Scale bar, 1 mm. **d**, A 0.5- μ m section of a spontaneous nodule from *dmi3-1* transformed with DMI3p-DMI3 1–311, in the absence of *S. meliloti*. Scale bar, 0.25 mm. **e**, Spontaneous nodules of DMI3p-DMI3 1–311 roots in the absence of *S. meliloti* show induction of *ENOD11-GUS*. Scale bar, 1 mm. **f**, **g**, Root hairs of *dmi3-1* roots transformed with DMI3p-DMI3 1–311 show no indication of deformation or formation of infection threads after inoculation with *S. meliloti* (**f**) in contrast with *dmi3-1* complemented by DMI3p-DMI3 (**g**). Scale bars, 40 μ m.

activated *ENOD11-GUS* in the absence of Nod factor (Fig. 2a, and Supplementary Fig. S2), indicating gain-of-function activities. Unlike the deletion constructs, DMI3 T271A and DMI3 T271D were able to complement *dmi3-1* (Fig. 2a), indicating that these gain-of-function mutants were also fully functional proteins. In the accompanying paper, Tirichine *et al.*⁹ show that mutation of the autophosphorylation residue (T265I) of *Lotus japonicus* CcCaMK leads to spontaneous nodulation when expressed from the native promoter. We would predict that DMI3 T271A and DMI3 T271D would also lead to spontaneous nodulation if expressed from the native promoter. Tirichine *et al.* demonstrate that T265I shows a loss of autophosphorylation and a decrease in substrate phosphorylation as has previously been reported for mutations at the equivalent residue of *L. longiflorum* CcCaMK¹⁰. Hence there seem to be different biochemical mechanisms underlying the gain of functions associated with deletion of the autoinhibition domain and mutation of the autophosphorylation site. The *L. japonicus* CcCaMK T265I mutation shows a mixture of empty spontaneous nodules and bacterium-infected nodules when inoculated with the symbiotic bacteria. This is consistent with our work showing that DMI3 T271A and T271D can act as gain-of-function proteins and in addition that they are fully functional proteins, unlike the deletion constructs.

Genetic analysis of nodulation mutants predicts that the receptor-like kinase DMI2 and the putative cation channel DMI1 lie upstream of both Ca^{2+} spiking and DMI3 in the nodulation signalling pathway^{11–14}. Consistent with these genetic predictions was our observation that 35S-DMI3 1–311 and 35S-DMI3 1–326 could activate *ENOD11-GUS* in *dmi1* and *dmi2* mutant lines (Fig. 3g, Supplementary Fig. S2 and Supplementary Table S1). Two members of the GRAS family of transcriptional regulators, NSP1 and NSP2, are proposed to function downstream of DMI3 (refs 12, 15–17). 35S-DMI3 1–311

and 35S-DMI3 1–326 did not induce *ENOD11-GUS* in the *nsp2-1* mutant but showed partial induction of *ENOD11-GUS* in the *nsp1-2* mutant (Fig. 3h, Supplementary Fig. S2 and Supplementary Table S1); GFP contained within the T-DNA was assayed for proof of transformation, and DMI3–GFP is stable and appropriately localized in these mutants (Supplementary Fig. S1). The *nsp1-1* mutant has been shown to have partial *ENOD11-GUS* induction after treatment with Nod factor¹². This work indicates that DMI3 lies upstream of NSP2 and most probably NSP1, and shows that the Ca^{2+} signal transmitted by DMI3 must act through these GRAS family transcriptional regulators.

The release of autoinhibition from CcCaMK is sufficient to activate nodule morphogenesis and the appropriate induction of early nodulation gene expression in the absence of bacterial elicitation. This demonstrates the essential function of CcCaMK in the regulation of nodule development and indicates that the activation of this protein through the oscillatory Ca^{2+} signal is necessary and sufficient for the induction of nodule morphogenesis. The fact that DMI3 1–311 and DMI3 1–326 are able to induce nodules, but are not sufficient to allow bacterial entry, indicates essential roles for the CaM-binding and EF-hand domains during rhizobial infection. It is possible that although the simple release of autoinhibition is sufficient to induce nodule development, the appropriate spatial and temporal activation of CcCaMK might be required during bacterial infection. This indicates that the role of Ca^{2+} oscillations might be to coordinate the plant's response with the bacterial responses to allow the appropriate and timely development of infection events. Our findings indicate that CcCaMK is a central regulator of nodule development, and a major question that we are currently addressing is whether the CcCaMK gain of functions can activate nodulation-like responses in non-legumes. The identification of an autoactivated form of CcCaMK may be a step towards the transfer of nodule development to non-legumes.

METHODS

Transformation of CcCaMK constructs. The CcCaMK constructs were generated by PCR with Gateway modified primers, using either DMI3 or *L. longiflorum* CcCaMK complementary DNA as template. A complete list of primers is provided in Supplementary Methods. The PCR products were cloned into the Gateway entry vector pDONR201 (Invitrogen) and recombined into either the destination vector pK7WG2D or a modified pK7WGF2 containing 1 kilobase upstream of the DMI3 start site, which is sufficient to drive DMI3 in complementation tests². Seedlings were transformed with *Agrobacterium rhizogenes* ARqua by following a standard procedure¹⁸. Plants were transferred to growth pouches one month after transformation, and nodules were scored two to four weeks later. For complementation, plants were inoculated with *S. meliloti* 1021 pXLGD4 (about 10^7 colony-forming units ml^{-1}). GFP fusions were generated by using in-frame versions of the above constructs and the destination vectors pK7WGF2 or pK7WGF2. GFP localization was analysed with 40 $\times$ 1.25 numerical aperture oil-immersion objective on an inverted Leica DM IRB confocal microscope.

For experiments involving pretreatment with Nod factor, plants were incubated for 6 h in buffered nodulation medium (BNM)¹⁹ with 1 nM Nod factor before staining for GUS²⁰. For cross-sections, 1-cm segments of stained roots were embedded in 3% agarose (Melford Laboratories) and sections (100–200 μ m) were prepared with a TAAB Series 1000 vibratome.

RNA extraction and RT-PCR. Transformed plants were incubated overnight in either BNM or BNM containing 1 nM Nod factor. The root systems of 7–14 plants were frozen in liquid nitrogen. Total RNA was isolated with Trizol reagent (Life Technologies) and treated with 2 units of Promega RQ1 DNase I. First-strand cDNA was synthesized with oligo(dT)₁₈ from 200 ng of RNA with the use of Superscript II reverse transcriptase (Invitrogen). The PCR cycle number was determined for each primer pair to the point at which amplification was in the linear phase.

Biochemical assays. Recombinant proteins containing His-tag fusions were expressed in *Escherichia coli* and purified to homogeneity with calmodulin-Sepharose chromatography (Amersham Biosciences) and/or Ni^{2+} -nitrilotriacetate agarose (Qiagen). The protein purity was checked by staining with Coomassie blue. The CaM-binding assay was performed as described²¹. The kinase assays were conducted as described previously⁵. GS peptide phosphorylation was measured with a P81 filter-binding assay as described in ref. 5.

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Author Contributions A.M. defined conditions and generated constructs for protein expression; S.C. and T.Y. did the *in-vitro* biochemistry; C.G. completed all the rest of the experimental work and wrote the paper with G.O.

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Deregulation of a Ca^{2+} /calmodulin-dependent kinase leads to spontaneous nodule development

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Induced development of a new plant organ in response to rhizobia is the most prominent manifestation of legume root-nodule symbiosis with nitrogen-fixing bacteria. Here we show that the complex root-nodule organogenic programme can be genetically deregulated to trigger *de novo* nodule formation in the absence of rhizobia or exogenous rhizobial signals. In an ethylmethane sulphonate-induced *snf1* (spontaneous nodule formation) mutant of *Lotus japonicus*, a single amino-acid replacement in a Ca^{2+} /calmodulin-dependent protein kinase (CCaMK) is sufficient to turn fully differentiated root cortical cells into meristematic founder cells of root nodule primordia. These spontaneous nodules are genuine nodules with an ontogeny similar to that of rhizobial-induced root nodules, corroborating previous physiological studies¹. Using two receptor-deficient genetic backgrounds we provide evidence for a developmentally integrated spontaneous nodulation process that is independent of lipochitin-oligosaccharide signal perception and oscillations in Ca^{2+} second messenger levels. Our results reveal a key regulatory position of CCaMK upstream of all components required for cell-cycle activation, and a phenotypically divergent series of mutant alleles demonstrates positive and negative regulation of the process.

Spontaneous nodule formation mutants of *Lotus* develop root nodules under rhizobia-free conditions (Fig. 1a). These spontaneous nodules are white and round; they lack the infection threads and bacteroid-containing cells that normally result from rhizobial invasion (Fig. 1b, c). Microscopical analysis show that *snf1-1* nodules, like wild-type rhizobial nodules, have peripheral vascular bundles (Fig. 1d–f), a globular shape, and attachment in root cortex (Fig. 1d, e), and develop from cortical cell divisions abutting vascular xylem poles (Fig. 1g–l). To investigate cellular ontogeny in *snf1-1* further, we took advantage of a *Nin*–GUS promoter fusion to trace the early cell divisions to nodule primordia opposite protoxylem poles (Fig. 1i, l).

When inoculated with *Mesorhizobium loti*, *snf1-1* mutants develop normal bacteria-filled nitrogen-fixing root nodules. In contrast, allelic loss-of-function mutants *ccamk-2* and *ccamk-3* are non-nodulating, whereas *ccamk-1* is leaky. In *ccamk-3* mutants treated with purified Nod-factor, both root hair swelling and root hair deformation were observed (not shown), indicating that Nod-factor perception initiating early cellular and physiological responses occurred. This conclusion was supported by Nod-factor-induced Ca^{2+} oscillations (Supplementary Fig. S1) in *ccamk-2* mutants, showing that a functional CCaMK gene is not required for the induction of Ca^{2+} spiking. A function downstream of Ca^{2+} spiking

was substantiated by an unchanged Nod-factor-dependent Ca^{2+} spiking in *snf1-1* mutant plants (Supplementary Fig. S1).

Legume roots are competent for rhizobial invasion in a zone where root hairs develop and respond maximally to Nod-factor. In inoculated wild-type plants, expression of *Nin*–GUS in root hairs and epidermal cells of this zone requires functional *Nfr1* and *Nfr5* Nod-factor receptors². We used this monitoring system to examine whether *snf1-1* mutants are constitutively activated for early symbiotic responses and to investigate the extent of cellular responses. Inoculation of *snf1-1* mutants carrying a *Nin*–GUS reporter resulted in an expression zone similar to that in *Nin*–GUS wild-type plants. Furthermore, *Nin*–GUS expression was not detected in uninoculated plants or plants inoculated with a Nod-factor-deficient *M. loti nodC* mutant (Fig. 1m–o). This indicates a susceptibility comparable to that of the wild-type and shows that *Nin*–GUS-detectable early signal transduction is dependent on Nod-factor. Later during spontaneous nodule organogenesis, expression was detected in primordial foci and in developing nodule organs in the same way as in rhizobial nodules (Fig. 1p, q). Ectopic expression in root cells was not detected.

Spontaneous nodule development in the absence of exogenous Nod-factor signalling predicts a mutant phenotype that is independent of the *Lotus* NFR1 and NFR5 receptors normally required for electrophysiological responses, Ca^{2+} spiking, root hair deformation and initiation of nodule primordia². To test this hypothesis we analysed the phenotype of *snf1-1 nfr1-1* double mutants: these form spontaneous root nodules in the same way as *snf1-1* mutants (Fig. 2a). *snf1-1* mutants develop up to eight spontaneous nodules, indicating that the conversion of cortical cells into nodule stem cells might be controlled or that subsequent organ development might be regulated. We tested this hypothesis in a hypernodulating *har1-1* genetic background³. In the absence of rhizobia, *snf1-1 har1-1* double mutants developed an average of 15.4 spontaneous nodules, whereas *snf1-1* mutants developed 6.6 nodules and *har1-1* developed none (Fig. 2b). These results indicate that only a few cells dedifferentiate and sustain cell divisions during the *snf1-1* nodule initiation process, which is still autoregulated. In accord with this observation, tissue culture of *snf1-1* explants revealed no gross changes in cell differentiation or phytohormone homeostasis (Supplementary Fig. S2).

Genetic mapping¹ identified the *Lotus* CCaMK gene as a homologue of lily⁴, tobacco⁵ and *Medicago*^{6,7} genes encoding CCaMK. Sequencing of *ccamk-1*, *ccamk-2*, *ccamk-3*, *ccamk-4* (refs 8–10) and *snf1-1* mutant alleles identified an allelic series. Single-nucleotide mutations led to a stop codon in *ccamk-2*, generating a truncated protein carrying one EF-hand, and to the substitution of Gly30 (G30E) in *ccamk-3* and Ser25 (S25F) in *ccamk-4* (Supplementary

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Table S1). A 299-base-pair duplication was found in *ccamk-1*. In *snf1-1*, C → T transitions led to the replacement of Thr 265 by isoleucine and a mutation in intron 1 (Fig. 3a, and Supplementary Table S1). The *Lotus* CCaMK gene comprises seven exons (Fig. 3a), and Southern hybridization indicates a single-copy gene.

To access each *snf1-1* mutation individually, transformation constructs carrying only the T265I substitution (*snf1-2*) or the intron mutation (*snf1-3*) were made by site-directed mutagenesis. Spontaneous nodulation was observed on *Agrobacterium rhizogenes* transgenic roots of *ccamk-2* mutants transformed with constructs carrying either *snf1-2* or both point mutations (*snf1-1*). Neither the intron 1

mutation (*snf1-3*) nor the wild-type gene conferred spontaneous nodulation (Supplementary Table S2b). All four constructs complemented *ccamk-2* for rhizobial nodulation, showing that they encode a CCaMK protein performing its positive function (Supplementary Table S2a, Fig. 2c). Spontaneous nodulation obtained with *snf1-2* constructs confirms that spontaneous organogenesis is caused by mutational change of CCaMK protein properties. Further support for this conclusion comes from the dominant-negative effect observed in *snf1-1* mutants transformed with the wild type or the *snf1-3* mutant (Supplementary Table S2c).

Expression of CCaMK is organ regulated. In comparison with leaves, stems and cotyledons, a 25-fold higher transcript level was found in uninoculated roots and in nodules (Fig. 3b). Expression is affected only marginally by inoculation with *M. loti* (Fig. 3c). To investigate whether the transcription of CCaMK was limiting nodulation, we determined transcript levels in hypernodulating *har1-5* *Lotus* mutants, in which nodule initiation and organogenesis are unrestricted by autoregulation. Only minor variations in expression between uninoculated and inoculated wild type on the one hand and *har1-5* on the other (Fig. 3d) indicate that transcription of CCaMK might not be upregulated in hypernodulating mutants and is unlikely to be a limiting factor in wild-type plants. Autoregulation of spontaneous nodulation revealed in *snf1-1 har1-1* double mutants (Fig. 2b) seems not to be a simple transcriptional regulation of CCaMK.

The conceptual CCaMK protein consists of 518 amino-acid residues (Fig. 4a, b, and Supplementary Fig. S3). A possible nuclear localization sequence is followed by a serine/threonine kinase domain¹¹, a calmodulin (CaM)-binding/autoinhibition domain and three, or possibly four, visinin-like EF-hands (Fig. 4a, b). The point mutation in *snf1-1* leads to the replacement of Thr 265 by isoleucine. In lily CCaMK, the corresponding Thr 267 is the only site

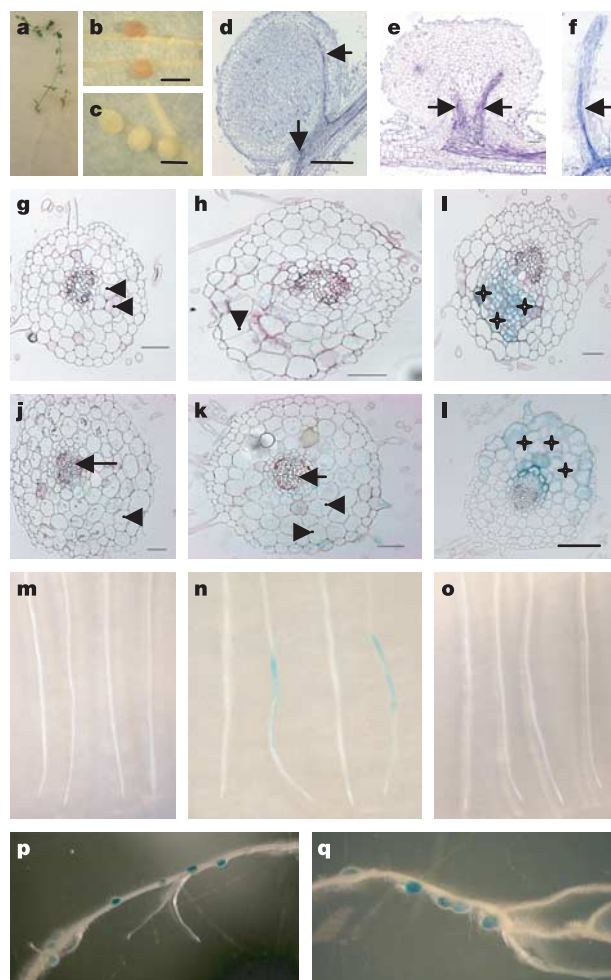


Figure 1 | Phenotype of *snf1-1* mutants and ontogeny of spontaneous nodules. **a**, *Lotus* wild-type and *snf1-1* (right). **b**, *M. loti* wild-type nodules. **c**, Spontaneous *snf1-1* nodules. **d**, Section of wild-type nodule. **e**, Spontaneous nodule. **f**, Lateral root. Black arrows indicate peripheral vascular bundles in nodules and central root vasculature. **g–i**, Transverse sections of wild-type *M. loti* nodule primordia. **g**, Initial cell division in the outer cortex; **h**, cell divisions in pericycle and cortex; **i**, later stage nodule primordium formed by the cell divisions in pericycle and cortex. In **g** and **h**, arrowheads indicate cells dividing opposite the xylem pole. **j–l**, Sections of spontaneous nodule primordia. **j**, Initial cell division in outer cortex; **k**, cells dividing in cortex and the pericycle; **l**, spontaneous nodule primordium formed by cells dividing in pericycle and cortex. In **j** and **k**, arrows indicate xylem poles; in **j** and **k**, arrowheads indicate pericycle and cortex cells dividing opposite xylem poles. **i**, **l**, Primordia cells are blue (stars) as a result of *Nin*-GUS activity. **m–o**, GUS activity in (from left to right) wild-type, wild-type *Nin*-GUS, *snf1-1* and *snf1-1 Nin*-GUS roots. **m**, Uninoculated. **n**, *M. loti*. **o**, *M. loti* *NodC*. **p**, **q**, *Nin*-GUS activity in wild-type rhizobial nodules (**p**) and spontaneous nodules (**q**). Scale bars, 1 mm (**b**, **c**); 100 μ m (**d**, **j**); 50 μ m (**h**, **k**, **l**); 20 μ m (**g**, **i**).

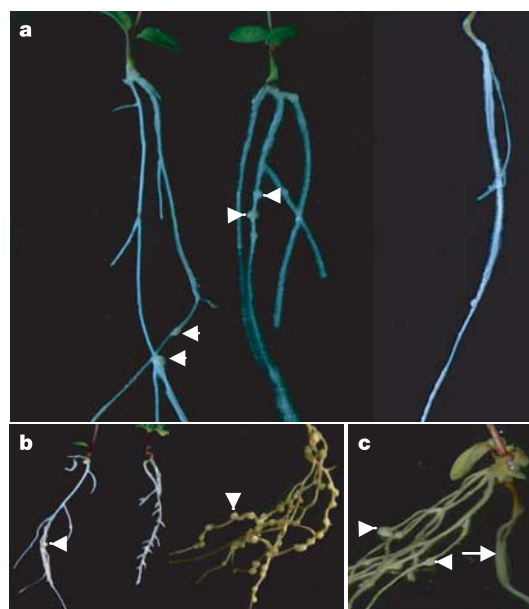


Figure 2 | Nod-factor receptor-independent development of spontaneous nodules and complementation of the *ccamk-2* loss of function mutation. **a**, From left to right: spontaneous nodules on *snf1-1* mutants, spontaneous nodules on *nfr1-1 snf1-1* double mutants, and *nfr1-1* control roots without nodules. **b**, From left to right: spontaneous nodules on *snf1-1* mutants, *har1-1* control roots without nodules, and spontaneous nodules on *snf1-1 har1-1* double mutants. Arrowheads indicate spontaneous nodules five weeks after germination. **c**, Spontaneous nodules on transgenic *ccamk-2* roots transformed with *snf1-1*. Arrowheads show hairy roots with spontaneous nodules. The arrow indicates the non-transgenic main root without spontaneous nodules.

for Ca^{2+} -dependent autophosphorylation^{12–14}. Studies *in vitro* show that lily CCaMK is activated by the binding of Ca^{2+} at EF-hands, causing increased affinity for CaM and release of autoinhibition, yielding a CCaMK with maximum substrate phosphorylation activity. This biochemical mechanism indicates that the primary activation of CCaMKs might occur in response to Ca^{2+} fluxes^{13–15}.

To determine the responsiveness of *Lotus* CCaMK to Ca^{2+} and CaM, wild-type CCaMK, G30E (*ccamk-3*) or T265I (*snf1-2*)-tagged proteins were purified from *Escherichia coli* and assayed *in vitro*. Wild-type CCaMK protein shows a twofold Ca^{2+} -stimulated increase in autophosphorylation, and substrate phosphorylation is accelerated by the addition of CaM (Fig. 4c, d). The level of *in vitro* autophosphorylation increased exponentially rather than linearly with increasing CCaMK concentration, indicating an intermolecular mechanism for autophosphorylation (Fig. 4e, and Supplementary Fig. S4). Autophosphorylation activities of G30E and T265I mutant proteins were undetectable (Fig. 4c). In a likely model, the positive regulatory role of CCaMK is triggered by Nod-factor-induced Ca^{2+} fluxes. This positive function is lost in *ccamk-2*, *ccamk-3* and *ccamk-4* mutants. However, the CCaMK T265I protein retains this positive regulatory role, indicating that a level of substrate phosphorylation activity lower than that in the wild-type (Fig. 4c), stimulated by Ca^{2+} and CaM, might be sufficient for nodulation.

Lack of autophosphorylation in the T265I mutant protein implies that autophosphorylation of CCaMK is dispensable for nodulation. The corresponding observation has been made for rhizobial nodulation of *Medicago truncatula*¹⁶. The fact that *snf1-1* is a recessive allele and the negative effect of the wild-type gene in *snf1-1* mutants (Supplementary Table S2c), indicate that wild-type CCaMK suppresses spontaneous *snf1-1* nodulation. CCaMK therefore seems to be under negative regulation. In *snf1-1* mutants negative regulation could be lost by conformational changes reminiscent of the signalling without kinase activity reported for an amino-acid-substituted tomato Pto (*Pseudomonas syringae* pv. *tomato* resistance) kinase¹⁷.

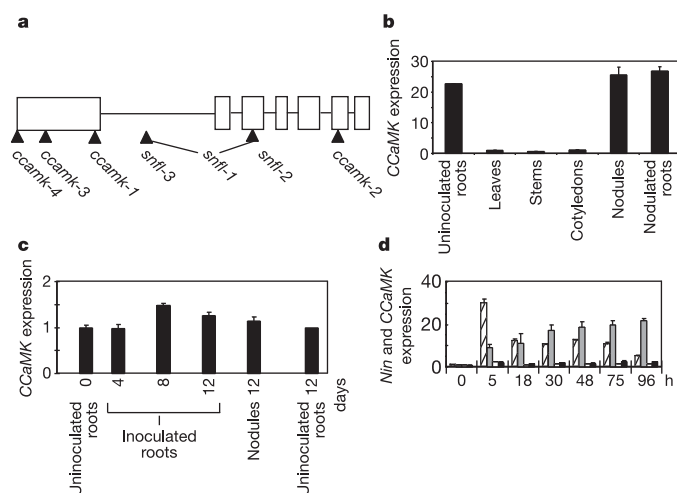


Figure 3 | The CCaMK gene and expression of CCaMK and *Nin* in wild-type and hypernodulation *har1-5* mutants. **a**, CCaMK gene structure. Open boxes, exons; lines, introns; black triangles, mutations in *snf1-1* and *ccamk-1* alleles. **b**, Steady-state CCaMK transcript level in root, leaf, stem, cotyledon, nodule and root segments from nodulated root. **c**, CCaMK transcripts in roots, roots after 12 days, roots 4–12 days after inoculation with *M. loti* and nodules at 12 days. **d**, CCaMK and *Nin* transcripts in wild-type and *har1-5* uninoculated roots, and roots inoculated for 5–96 h. Hatched columns, *Nin* expression in wild type; grey columns, *Nin* expression in *har1-5*; open columns, CCaMK expression in wild type; black columns, CCaMK expression in *har1-5*. Results are fold increase compared with leaves (**b**) and uninoculated roots (**c**, **d**). Error bars represent standard deviations from three independent experiments.

Alternatively, the autophosphorylation-induced inactivation associated with lily CCaMK¹⁵ protein aggregation could act negatively. It is unclear whether this aggregation occurs *in vivo*, but according to this mechanism the activity of CCaMK might be determined by the balance between activation by phosphorylation and aggregation. Lack of autophosphorylation in *snf1-1* and *snf1-2* leaves CCaMK in a ground state where activity is minimal but sufficient to trigger downstream signal transduction arising from a lack of autophosphorylation-dependent aggregation. According to this model, basal autophosphorylation in the wild type is sufficient for aggregation and CCaMK inactivation. Absence of autophosphorylation and aggregation in lily CCaMK, where Thr267 is replaced by alanine, supports this hypothesis¹⁵. Spontaneous nodulation of *M. truncatula* with decreased CCaMK activity supports the notion that a CCaMK activity that is less than maximal triggers downstream events¹⁶.

Intermolecular autophosphorylation is indicated by our *in vitro*

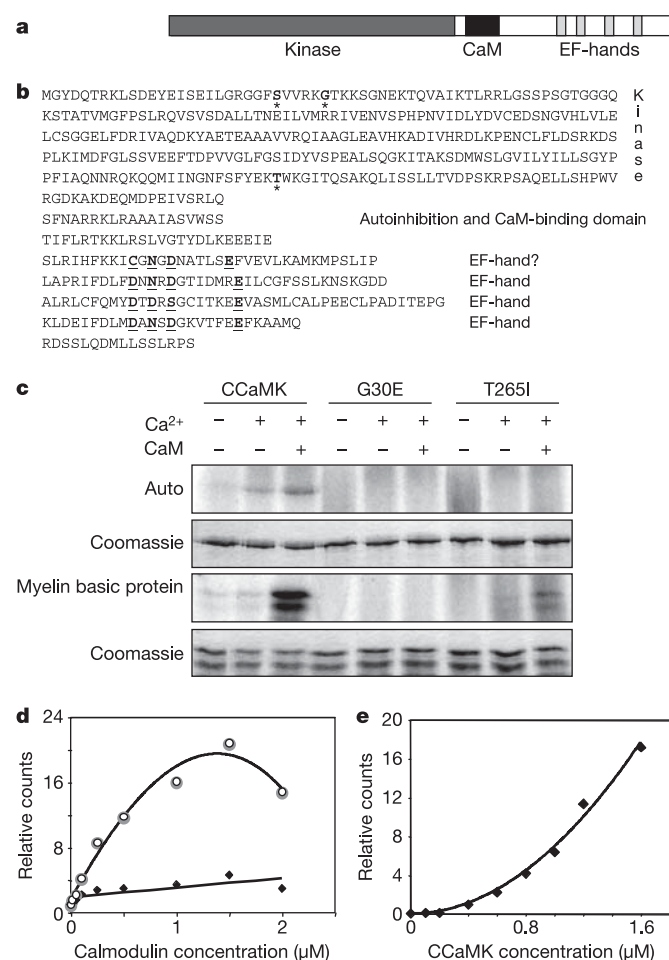


Figure 4 | Domains and *in vitro* kinase activity of CCaMK protein.

a, CCaMK domains. **b**, Amino acids arranged in protein domains. Bold residues with asterisk, autophosphorylation sites and amino acids substituted in mutants; bold underlined residues, conserved EF-domain residues. **c**, Autophosphorylation (auto) and substrate phosphorylation (myelin basic protein) of CCaMK, G30E and T265I proteins in the presence and absence of Ca^{2+} and CaM. Corresponding Coomassie staining is also shown as indicated. **d**, Effects of CaM on CCaMK autophosphorylation and substrate phosphorylation. Radioactivity incorporated into CCaMK protein or substrate was quantified and is presented relative to signal without CaM. Diamonds, autophosphorylation; circles, phosphorylation of myelin basic protein. **e**, Protein concentration-dependent autophosphorylation. Radioactivity incorporated into CCaMK is presented relative to a sample containing 0.4 μM CCaMK.

assays, and data from lily imply that active CCaMK is a multimeric enzyme¹⁴. This is consistent with the recessive nature of *snf1-1* and the dominant-negative effect of wild-type CCaMK in transgenic *snf1-1* (Supplementary Table S2c). Heterozygotes and hemizygotes would still make multimeric protein with sufficient wild-type subunits to behave in the same way as wild-type CCaMK. Supporting this notion is the observation that *snf1-1* shows penetrance in about 2.5% of heterozygous *wt/snf1-1* plants that develop spontaneous nodules, conceivably after the assembly of multimeric complexes with sufficient T265I subunits to escape negative regulation.

We show that CCaMK is necessary and sufficient for dedifferentiation of root cortical cells into nodule initials. Spontaneous nodulation of *nfr1-1 snf1-1* double mutants is direct evidence for Nod-factor-independent organ development resulting from a single amino-acid substitution. This provides compelling evidence for a pivotal role of mechanisms regulating CCaMK activity, and we infer that any parallel signal transduction pathways downstream of Nod-factor perception, or other signals from rhizobia required for the initiation of nodule primordia, seem to converge at or above CCaMK activation. Our results support a direct signal transduction mechanism from Nod-factor perception to meristem formation. This identification of the genetic switch for nodule organogenesis has significant biotechnological potential.

METHODS

Plant material. All mutants described are in the ecotype Gifu B-129 background. The *snf1-1* allele was isolated¹ after mutagenesis with ethylmethane sulphonate¹⁸, and *ccamk-1*, *ccamk-2*, *ccamk-3* and *ccamk-4* were previously called *sym15-1*, *sym15-2* (ref. 8), *sym72-1* and *sym72-2* (ref. 9). Plants were grown with or without *M. loti*, strain NZP2235 or MAFF30-3099.

Microscopic analysis. Nodules and root tissues were prepared for microscopic analysis as described previously¹.

Complementation. For *in planta* complementation, a genomic fragment containing the CCaMK gene including a 3.1-kilobase promoter region and a 3' region of 1.3 kilobases was inserted into the pIV10 plasmid. Triparental transfer of construct into *A. rhizogenes* strain AR12, and AR1193 and 'hairy root' transformation were performed as described previously².

Other methods. The double-mutant analysis, *Nim*-GUS reporter lines, expression analysis and *in vitro* kinase assays are described in Supplementary Methods S5.

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Author Information The sequences for the *L. japonicus* ecotype GIFU CCaMK gene and mRNA are deposited in the EMBL nucleotide database under accession numbers AM230792 and AM230793. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.S. (stougaard@mb.au.dk).

Mitochondrial dysfunction in *Drosophila* *PINK1* mutants is complemented by *parkin*

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Autosomal recessive juvenile parkinsonism (AR-JP) is an early-onset form of Parkinson's disease characterized by motor disturbances and dopaminergic neurodegeneration^{1,2}. To address its underlying molecular pathogenesis, we generated and characterized loss-of-function mutants of *Drosophila* *PTEN-induced putative kinase 1* (*PINK1*)³, a novel AR-JP-linked gene⁴. Here, we show that *PINK1* mutants exhibit indirect flight muscle and dopaminergic neuronal degeneration accompanied by locomotive defects. Furthermore, transmission electron microscopy analysis and a rescue experiment with *Drosophila* *Bcl-2* demonstrated that mitochondrial dysfunction accounts for the degenerative changes in all phenotypes of *PINK1* mutants. Notably, we also found that *PINK1* mutants share marked phenotypic similarities with *parkin* mutants. Transgenic expression of *Parkin* markedly ameliorated all *PINK1* loss-of-function phenotypes, but not vice versa, suggesting that *Parkin* functions downstream of *PINK1*. Taken together, our genetic evidence clearly establishes that *Parkin* and *PINK1* act in a common pathway in maintaining mitochondrial integrity and function in both muscles and dopaminergic neurons.

The *Drosophila* *PINK1* gene (also called CG4523) encodes a polypeptide of 721 amino acids with a molecular mass of about 80 kDa (Fig. 1c and Supplementary Fig. S1a). Similar to human *PINK1* (ref. 4), structural analysis of *Drosophila* *PINK1* protein also revealed two characteristic motifs: a mitochondrial targeting motif and a serine/threonine kinase domain. The kinase domain exhibited 60% similarity (42% identity) to that of human *PINK1*. Consistent with the localization of human *PINK1* (ref. 4), *Drosophila* *PINK1* was also found localized in mitochondria (Supplementary Fig. S1b, c).

First, we observed the expression patterns of *PINK1*, and found that its transcripts are ubiquitously expressed throughout all developmental stages (Supplementary Fig. S1d). Its spatial expression in adults was broadly distributed over all segments, but was particularly higher in the thorax (Supplementary Fig. S1e). To reveal *in vivo* roles of *PINK1* in *Drosophila*, we generated *PINK1* loss-of-function mutant flies, *PINK1*^{D3} and *PINK1*^{B9}, as well as revertants (*PINK1*^{RV}) (Fig. 1a), and these alleles were confirmed by conducting Southern, northern and western blot analyses and genetic analysis with *PINK1* RNA-mediated interference lines (Supplementary Fig. S1f, g, Fig. 1b, c and Supplementary Fig. S2, respectively). *PINK1* mutants were viable and developed to adulthood; however, they displayed shorter longevity (Supplementary Fig. S3a) and complete male sterility due to impaired sperm with swelled nebenkern, a specialized mitochondrial derivative (Fig. 1d). Moreover, at the age of 3 days, about 65% of *PINK1* mutants exhibited a downturned wing phenotype with rigidity, and this percentage slightly increased thereafter (Fig. 1e and Supplementary Fig. S3b). To

confirm whether this wing phenotype is caused by loss of *PINK1* function, we generated UAS-*HA-PINK1* transgenic lines. When *PINK1* expression was induced using the *hs-GAL4* driver in the *PINK1* mutant background, this wing phenotype was remarkably restored (Fig. 1e and Supplementary Fig. S3b). The discovery of the downturned rigid wing phenotype in *PINK1* mutants led us to search for locomotion defects in these flies. As expected, they showed complete defects in flight ability and slower climbing speed, and these phenotypes were all rescued by *PINK1* expression (Fig. 1f and g, respectively).

Furthermore, we found another apparent phenotype in the thoraces of *PINK1* mutants right after eclosion (~85%); thoraces were crushed, particularly in the mid-anterior and anterolateral regions (Fig. 1h, i), and the percentage of flies with this crushed thorax phenotype progressively increased over time (~95% at the age of 30 days, Fig. 1i). Cross-thoracic sections of *PINK1* mutants showed abnormal structure and reduced content of indirect flight muscle (Fig. 2a, top row). In the magnified sections of the mutants, we found disorganized muscle fibres and enlarged mitochondria with highly decreased staining intensity (Fig. 2a, bottom row). Consistently, examination of the indirect flight muscle ultrastructure of *PINK1* mutants revealed irregular arrangement of myofibrils and immensely swollen mitochondria with loss of the outer membrane (Fig. 2b and Supplementary Fig. S3c). Given the reduced muscle content and mitochondrial impairment in the mutants, we then investigated whether apoptosis is apparent in the muscles of *PINK1* mutants by performing a TdT-mediated dUTP nick end labelling (TUNEL) assay. TUNEL signals were ubiquitously detected in the indirect flight muscle of *PINK1* mutants, but not in control flies (Fig. 2c). These *PINK1* mutant phenotypes were all rescued by *PINK1* expression (Figs 1i and 2a, c). Collectively, these results demonstrate that loss of *PINK1* induces indirect flight muscle degeneration and mitochondrial impairment.

To confirm further mitochondrial impairment in the muscles (Fig. 2b), we quantified mitochondrial abundance in the mutants. As expected, a marked reduction in the levels of mitochondrial DNA (mtDNA) and protein was observed in *PINK1* mutants when compared to the controls (Fig. 2d and Supplementary Fig. S4a, respectively), and these levels were almost completely restored when *PINK1* was expressed back in the mutants (Fig. 2d and Supplementary Fig. S4a). We next conducted an ATP quantification assay with the thoraces of the mutant flies to determine whether altered mitochondrial morphology and amount are correlated with its functional loss. Compared to the control flies, *PINK1* mutants showed a more than twofold reduction in ATP level, and this was markedly restored by *PINK1* expression (Fig. 2e). Because substantial

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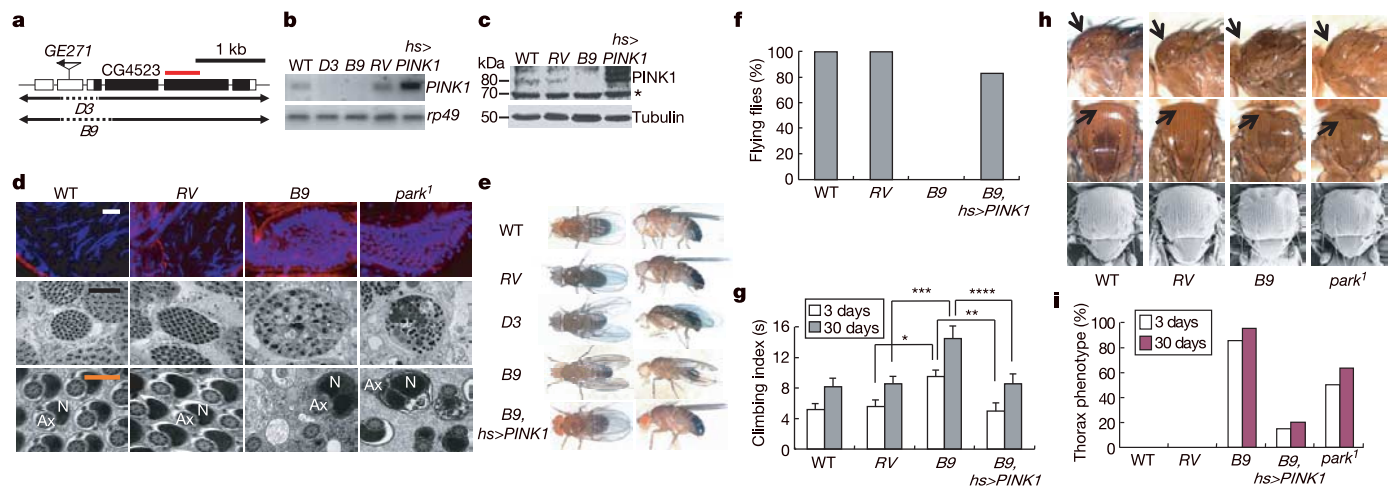


Figure 1 | Characterization of *PINK1* mutants. **a**, Exons of *PINK1* (CG4523) are indicated by boxes, and coding regions are coloured black. The deleted regions for D3 (*PINK1*^{D3}, 379 bp) and B9 (*PINK1*^{B9}, 570 bp) are also presented. The red bar indicates the region used as a probe for northern blotting. **b**, **c**, Determination of the expression level of *PINK1* by northern (b) and western (c) blotting. Asterisk indicates a nonspecific band. **d**, Immunostaining of the nucleus of sperm in 3-day-old male testis (blue, Hoechst 33258; red, α -tubulin; top panels). TEM analysis of spermatozoa from the testis of males hatched within 12 h (middle and bottom panels).

Ax, axoneme; N, nebenkern. White scale bar, 5 μ m; black scale bar, 5 μ m; orange scale bar, 500 nm. **e**, Downturned wing phenotypes of *PINK1* mutants. **f**, Comparison of flight ability. **g**, Comparison of climbing rate (asterisk, $P = 4.59 \times 10^{-4}$; two asterisks, $P = 1.91 \times 10^{-3}$; three asterisks, $P = 1.70 \times 10^{-6}$; four asterisks, $P = 3.46 \times 10^{-5}$). Error bars indicate mean $\pm$ s.d. **h**, Collapsed-thorax phenotypes (black arrows) of *PINK1* mutants right after eclosion. **i**, Percentage of collapsed-thorax phenotypes. Details of all the indicated genotypes in this and other figures are described in Supplementary Information.

evidence indicated that anti-apoptotic Bcl-2 families are involved in the protection of mitochondrial integrity and function^{5,6}, we hypothesized that Bcl-2 expression may suppress the mitochondrial dysfunction and other apparent phenotypes of *PINK1* mutants. As a result, a remarkable recovery of the levels of mtDNA (Fig. 2d), mitochondrial protein (Supplementary Fig. S4a) and ATP (Fig. 2e) was observed after expression of Buffy—a sole *Drosophila* Bcl-2 homologue⁷—under the control of the *hs*-GAL4 driver, whereas its expression in the wild-type background did not affect the mitochondria (Supplementary Fig. S4b–d). Consistently, all of the *PINK1* mutant phenotypes except for the flight defects were markedly restored by Buffy expression (Fig. 2a, f and Supplementary Fig. S4e, f). Overall, these results strongly suggest that mitochondrial dysfunction is the main cause of these aberrant phenotypes of *PINK1* mutants.

Because dopaminergic neurodegeneration is one of the major characteristics of AR-JP patients², we examined whether dopaminergic neurons in the brains of adult mutant flies are also impaired. Using immunostaining with tyrosine hydroxylase (TH) antibody, the number of dopaminergic neurons was counted in a blind fashion. For *PINK1* mutants, the number of dopaminergic neurons within each of the major clusters—including dorsomedial (DM), dorsolateral (DL) 1 and 2, and posteriomedial (PM) clusters—was not changed at 3 days (data not shown). However, at 30 days, the mutants exhibited a small but significant decrease ($\sim 10\%$) in the number of dopaminergic neurons in the DM and DL1 regions compared with the wild-type and revertant flies (Fig. 3a, b). This was further confirmed by using another method of expressing nuclear LacZ in dopaminergic neurons (also done in a blind fashion; Supplementary Fig. S5a, b). In

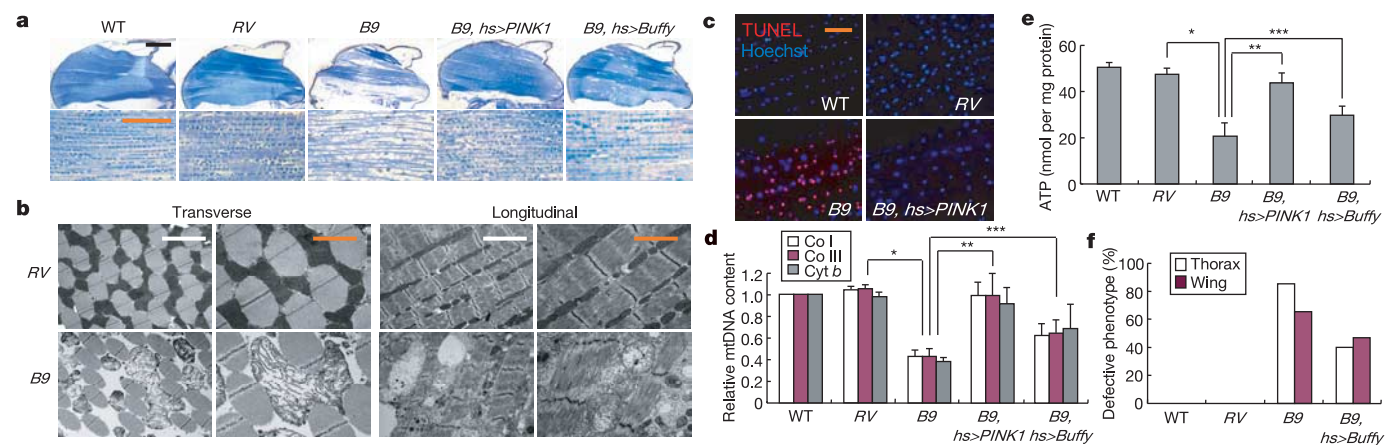


Figure 2 | Mitochondrial defects in *PINK1* mutants. **a**, Longitudinal sections of thoraces. Black scale bar, 200 μ m; orange scale bar, 20 μ m. **b**, TEM analysis of indirect flight muscle of 2-day-old males. White scale bar, 5 μ m; orange scale bar, 2 μ m. **c**, Merged images of apoptotic cells (TUNEL, red) and nuclei (Hoechst 33258, blue) of the indirect flight muscle. Scale bar, 20 μ m. **d**, Quantification of the mtDNA of thoraces (only the *P* values for cytochrome *c* oxidase subunit III are shown: asterisk, $P = 1.21 \times 10^{-3}$; two

asterisks, $P = 9.60 \times 10^{-3}$; three asterisks, $P = 4.47 \times 10^{-2}$; $n = 3$). Co I, cytochrome *c* oxidase subunit I; Co III, cytochrome *c* oxidase subunit III, Cyt b, cytochrome *b*. **e**, Comparison of the ATP content of thoraces (asterisk, $P = 7.20 \times 10^{-4}$; two asterisks, $P = 2.10 \times 10^{-3}$; three asterisks, $P = 3.08 \times 10^{-2}$). **f**, Percentage of defective thorax and wing phenotypes. Error bars in **d**, **e** indicate mean $\pm$ s.d.

addition to this neuronal loss at 30 days, the mutants exhibited a markedly decreased anti-TH staining intensity in their dopaminergic neurons (Fig. 3a), which was consistent with their reduced level of dopamine (Fig. 3c). To examine whether the cause of this phenotype is correlated with mitochondrial impairment, we generated *TH>mitoGFP* lines to express mitochondria-targeted green fluorescent protein (GFP) in dopaminergic neurons. In *PINK1* mutants, a number of mitochondria were found to be profoundly enlarged in dopaminergic neurons in all of the clusters, the most severe of which were in the DL1 cluster (Fig. 3d, e and Supplementary Fig. S5c, d), and mitochondrial size progressively increased with age

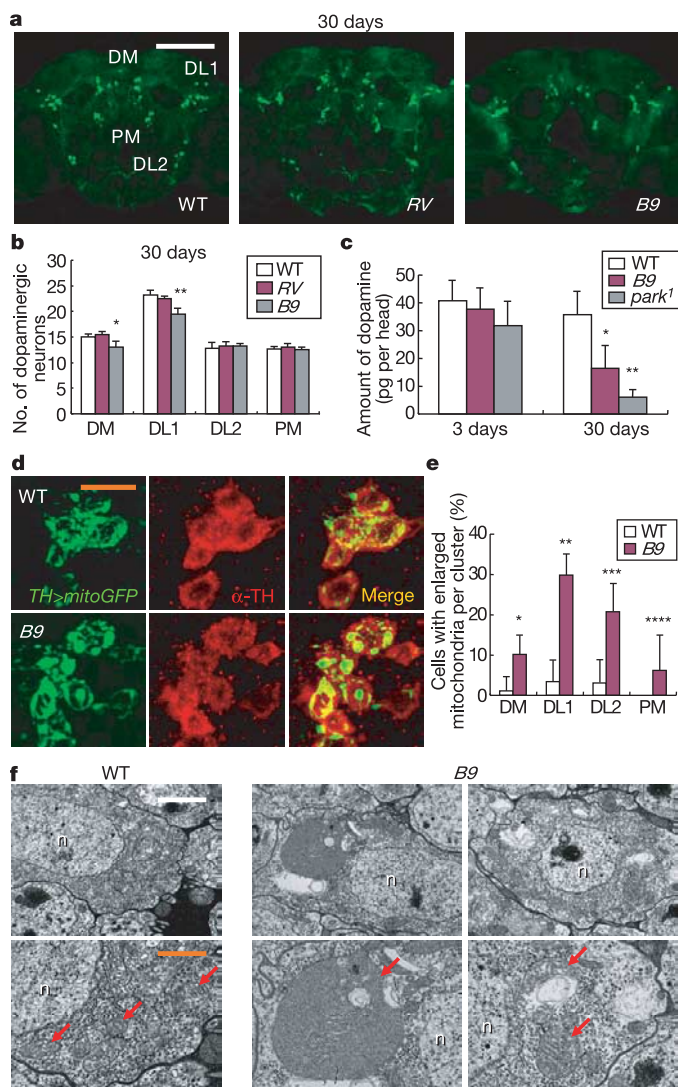


Figure 3 | Dopaminergic neuronal degeneration in *PINK1* mutants.

a, Whole-mount adult male brains (30-day-old) showing dopaminergic neuron clusters marked by anti-TH antibody (green). Scale bar, 100 μ m. **b**, Graph showing the number of dopaminergic neurons in each cluster at 30 days (asterisk, $P = 3.68 \times 10^{-8}$; two asterisks, $P = 2.73 \times 10^{-9}$; $n = 20$). **c**, Amount of dopamine in adult head (asterisk, $P = 6.85 \times 10^{-3}$; two asterisks, $P = 1.19 \times 10^{-3}$; $n = 4$). **d**, Examination of the mitochondria in dopaminergic neurons within the DL1 cluster of 3-day-old adult brain. Scale bar, 10 μ m. **e**, Graph showing the percentage of the number of dopaminergic cells with mitochondria larger than 2 μ m in diameter over the total number of dopaminergic cells in each cluster (asterisk, $P = 1.13 \times 10^{-5}$; two asterisks, $P = 2.00 \times 10^{-8}$; three asterisks, $P = 9.36 \times 10^{-6}$; four asterisks, $P = 3.83 \times 10^{-2}$; $n = 10$). **f**, TEM analysis of the dopaminergic neurons in the DL1 cluster of 20-day-old adult brain. Red arrows indicate the mitochondria in dopaminergic neurons. n, nucleus. White scale bar, 2 μ m; orange scale bar, 1 μ m. Error bars in **b**, **c**, **e** indicate mean $\pm$ s.d.

(data not shown). Consistent with this result, transmission electron microscopy (TEM) analysis also revealed grossly enlarged mitochondria in dopaminergic neurons of the mutants (Fig. 3f). In addition, we observed enlarged mitochondria in other neurons, such as surrounding cell bodies of dopaminergic neurons in posterior protocerebrum and neuropils (Supplementary Fig. S6 panels a and b, respectively), and serotonergic neurons (Supplementary Fig. S6d), but not in photoreceptor cells, insulin-secreting cells and circadian pacemaker cells (Supplementary Fig. S6 panels c, e and f, respectively). These results indicate that *PINK1* is a critical factor required in dopaminergic neurons for maintaining mitochondrial integrity as well as neuronal function.

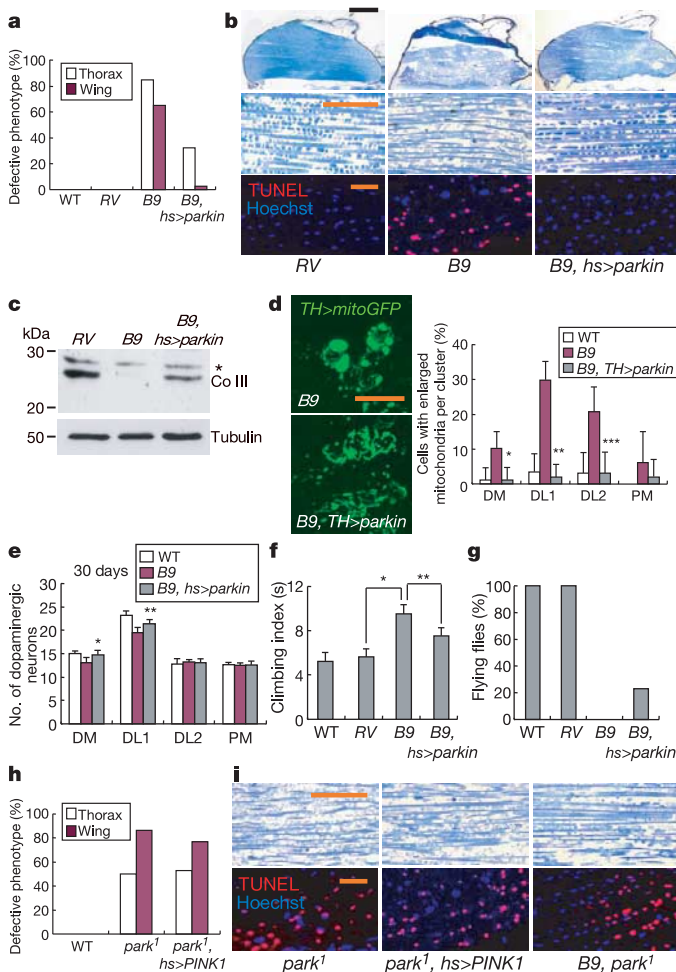


Figure 4 | In vivo interaction between *PINK1* and *Parkin*.

a, Percentage of defective thorax and wing phenotypes. **b**, Longitudinal sections of thoraces (top and middle panels), and merged images (bottom panels) of TUNEL (red) and Hoechst 33258 (blue) staining of the thoraces. Black scale bar, 200 μ m; orange scale bar, 20 μ m. **c**, Western blot of mitochondrial protein cytochrome c oxidase subunit III (Co III) in thoraces ($n = 3$). **d**, Examination of mitochondria in dopaminergic neurons within the DL1 cluster of 3-day-old adult brain (left). Scale bar, 10 μ m. Graph showing the percentage of the number of dopaminergic cells with mitochondria larger than 2 μ m in diameter over the total number of dopaminergic cells in each cluster (right; asterisk, $P = 5.38 \times 10^{-4}$; two asterisks, $P = 6.32 \times 10^{-10}$; three asterisks, $P = 4.79 \times 10^{-5}$; $n = 10$). **e**, Graph showing the number of dopaminergic neurons in each cluster at 30 days (asterisk, $P = 3.51 \times 10^{-4}$; two asterisks, $P = 1.04 \times 10^{-5}$; $n = 20$). **f**, Comparison of the climbing rate (asterisk, $P = 4.59 \times 10^{-4}$; two asterisks, $P = 2.40 \times 10^{-2}$). **g**, Comparison of flight ability. **h**, Percentage of defective thorax and wing phenotypes. **i**, Longitudinal sections of thoraces (top panels), and merged images (bottom panels) of TUNEL (red) and Hoechst 33258 (blue) staining of the thoraces. Scale bar, 20 μ m. Error bars in **d-f** indicate mean $\pm$ s.d.

We noticed that all of the phenotypes of the *PINK1* mutants were highly reminiscent of *parkin* mutants generated by other groups and us^{8–11}: abnormally positioned wings (Supplementary Fig. S3b), crushed thoraces (Fig. 1h, i), disorganized muscle fibres with enlarged mitochondria (Fig. 4i), muscle cell apoptosis (Fig. 4i), impaired flight ability and highly reduced climbing rate (Supplementary Fig. S8 panels a and b, respectively), complete male sterility due to defective nebenkern (Fig. 1d), and defects in dopaminergic neurons in two particular clusters, DL1 (ref. 10) and DM (refs 8, 9). Therefore, we tested whether there are any possible interactions between these two genes by first expressing Parkin in *PINK1* mutants. The crushed thorax and downturned wing phenotypes of *PINK1* mutants were markedly restored by Parkin expression (Fig. 4a). Muscle sections showed intact structure of muscles and mitochondria in Parkin-expressed *PINK1* mutants (Fig. 4b). Moreover, the mtDNA and mitochondrial protein content as well as the level of ATP in the indirect flight muscle were markedly rescued by Parkin expression (Fig. 4c and Supplementary Fig. S7a, b). Consistent with these findings, TUNEL signals in the indirect flight muscle were almost completely absent (Fig. 4b). In addition, the loss of dopaminergic neurons in the DM and DL1 regions of the *PINK1* mutants was significantly restored by Parkin expression (Fig. 4e), and the enlarged mitochondria in the dopaminergic neurons within the DM, DL1 and DL2 clusters were also markedly rescued (Fig. 4d). Furthermore, the rescued flies showed increased climbing speed and were even able to fly (Fig. 4 panels f and g, respectively). These results indicate that Parkin can compensate for both the mitochondrial dysfunction produced by loss of *PINK1* in muscles and dopaminergic neurons, and for the consequent defective phenotypes. Furthermore, we found that Parkin could not block apoptosis induced by well-known cell death molecules (Supplementary Fig. S7c), suggesting that suppression of *PINK1* loss-of-function phenotypes by Parkin expression is not a result of its general protective role against apoptotic insults but rather results from its specific protective role against mitochondrial dysfunction induced by loss of *PINK1*.

Genetic analysis showed that the apparent phenotype as well as the mitochondrial morphology and apoptosis in *parkin* mutants could not be recovered by increased *PINK1* expression (Fig. 4h, i and Supplementary Fig. S8a, b). These results indicate that Parkin acts downstream of *PINK1* to maintain mitochondrial integrity and function. Moreover, we generated and observed *PINK1* and *parkin* double mutants, and found that they do not show further severity in the phenotype (Fig. 4i and Supplementary Fig. S8c, d) compared to either of the single mutants, implicating that *PINK1* and Parkin indeed converge in a common genetic pathway. Furthermore, mitochondrial localization of both of these molecules supports our idea of their functional interaction in that organelle (Supplementary Fig. S8e, f).

We have previously demonstrated that inactivation of the JNK pathway prevents impaired morphology and decreased staining intensity of dopaminergic neurons in *parkin* mutants⁸. Together with the knowledge of the *in vivo* interaction between *PINK1* and Parkin, we hypothesized that the JNK pathway may be activated in the indirect flight muscle of *PINK1* mutants. Indeed, using a *puc-lacZ* line (where *puc* is a JNK target gene¹²), we found strong ectopic *puc* expression in the muscles of the *PINK1* mutants, but not in the wild-type flies (Supplementary Fig. S8g). Furthermore, we generated *hep* (a *Drosophila* MKK7 homologue¹³) and *PINK1* double mutants, and found that they show significant restoration of the apparent phenotypes and markedly reduced apoptosis (Supplementary Fig. S8h, i), whereas the mitochondria remain swelled (Supplementary Fig. S8i). Collectively, these data suggest that the mitochondrial impairment-induced apoptosis resulting from *PINK1* ablation is mediated by ectopic activation of the JNK pathway.

Our study provides genetic evidence that *PINK1* has a key role in mitochondria, and its dysfunction contributes to the degeneration of high-energy-demanding cells including dopaminergic neurons,

muscles, as well as sperm. However, there seems to be some variation in the level of vulnerability to mitochondrial impairment among the tissues and dopaminergic neuron clusters, which might be explained by the different threshold levels for mitochondrial dysfunction-induced cell death among the types of tissues and cells.

All of the phenotypes displayed by *PINK1* mutants were strikingly similar to those of *parkin* mutants. The results of the rescue experiment with the *parkin* transgene provide definitive evidence for the functional interaction between *PINK1* and Parkin, and this indicates that Parkin also has a crucial role in maintaining mitochondrial integrity and function. Moreover, through epistatic analysis, Parkin was found to act downstream of *PINK1*. This finding of the convergence of Parkin and *PINK1* in a pathway involved in the protection of mitochondrial integrity provides invaluable clues for understanding the central pathogenic mechanism of AR-JP. Taken together, we anticipate that our *Drosophila* system will be highly useful in future studies, such as investigation into how *PINK1* and Parkin protect mitochondrial integrity, and identification of the upstream and downstream molecules involved in this pathway. Our findings will lead to the development of better and effective treatment strategies for AR-JP and possibly other forms of Parkinson's disease, which should be aimed specifically against mitochondrial dysfunction.

METHODS

Fly stocks. From the GenExel library, we isolated a P-element insertion line (*PINK1*^{GE271}) in the second exon of the gene (Fig. 1a). *PINK1*^{D3} and *PINK1*^{B9} alleles were generated through imprecise excision of the P element of *PINK1*^{GE271} and found to be loss-of-function mutants for *PINK1* (Fig. 1b, c). We also generated a revertant allele (*PINK1*^{RV}) by precise excision (but has 40 bp of the P element), which showed almost the same amount of transcript and protein as that of the wild type (Fig. 1b, c). The generation of *park*¹ mutants and UAS-*parkin* has been described previously⁸. The UAS-*Buffy* fly line was a gift from H. Richardson⁷; the UAS-*mitoGFP* line was from H. J. Bellen¹⁴ (generated by A. Pilling and W. Saxton). The *TH-GAL4* fly line was a gift from S. Birman¹⁵. The *hs-GAL4* line was obtained from the Bloomington Stock Center.

Northern blotting, mtDNA PCR and immunoblot analysis. Northern blotting was conducted as previously described¹⁶ with purified mRNA using a 504-bp fragment of *PINK1* open reading frame (Fig. 1a) as a probe. For mtDNA PCR, total DNA of thoraces of 2-day-old flies was extracted and subjected to PCR. The genomic DNA level of *rp49* of each sample was also examined by PCR and used as a loading control. Results are expressed as fold change relative to the control. Immunoblot analysis was conducted as previously described¹⁷ with the thoraces of 2-day-old flies. Mouse monoclonal anti-cytochrome *c* oxidase subunit III (yeast) antibody (Molecular Probes), anti- β -tubulin (E7) mouse antibody (Developmental Studies Hybridoma Bank (DSHB)) and rabbit anti-*PINK1* antibody were used at 1:1,000 dilutions. The polyclonal antibody to *Drosophila* *PINK1* was generated in rabbit by injecting glutathione S-transferase (GST)-fused *PINK1* (amino acids 480–960) and further purified.

Immunostaining and TUNEL assay. Adult brain and testis were fixed with 4% paraformaldehyde and blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) with 2% bovine serum albumin. The primary antibodies used in this study were: anti-TH rabbit antibody (1:50, Pel-Freez) and anti- α -tubulin mouse antibody (1:100, DSHB). Hoechst 33258 (Sigma) was used to visualize the nucleus of sperm. For adult brains, Z-series images (images for $\times 200$ were sectioned at 1- μ m intervals, and $\times 1,000$ or $\times 3,000$ at 0.3 μ m) were obtained by LSM510 confocal microscope. For the TUNEL assay, apoptosis in the thoraces of 2-day-old flies was detected using the *in situ* cell death detection kit (Roche).

Muscle section and TEM. Muscle sections were carried out as previously described¹¹, but with some modifications. The samples embedded in Spurr's resin were trimmed and sectioned from the lateral side of the thorax (at a thickness of 5 μ m between 150 μ m and 300 μ m in depth), and the serial sections were then stained with Toluidine blue dye. About ten thoraces of 2-day-old flies were observed for each genotype. The sections were observed at two magnifications ($\times 100$ and $\times 1,000$) for light microscopy (Leica). For TEM analysis, samples were prepared as previously described¹⁸.

ATP assay. Five thoraces from 2-day-old flies were dissected and homogenized in 100 μ l of 6 M guanidine-HCl in extraction buffer (100 mM Tris and 4 mM EDTA, pH 7.8) to inhibit ATPases¹⁸, followed by fast-freezing in liquid nitrogen and boiling for 3 min. The samples were then centrifuged to collect the supernatant, which was then diluted (1/750) with extraction buffer and mixed

with luminescent solution (Enliten kit, Promega). The luminescence was measured by a luminometer (Berthold Technologies), and the results were compared to the standards. The relative ATP level was then calculated by dividing the luminescence by the total protein concentration, which was determined by the Bradford method. For Bradford assays, samples were diluted (1/30) with extraction buffer. Average $\pm$ s.d. is from $n = 3$ experiments.

Dopamine enzyme immunoassay. Dopamine enzyme immunoassay (LDN) was conducted according to the manufacturer's instruction, but fly samples were prepared as follows: 50 fly heads per genotype were dissected and homogenized in PBS with assay buffer (1 M HCl). Then, after adding extract buffer, they were incubated for 20 min and then centrifuged at 13,000 r.p.m. for 30 min. The supernatant was collected and assayed.

Behavioural assays. For the assay of climbing speed, groups of ten 3-day-old males were transferred into 18-cm-long vials and incubated for 1 h at room temperature for environmental acclimatization. After tapping the flies completely down to the bottom, we marked their climbing time at the 15-cm finish line when more than five flies had arrived. Five trials were performed for each group and repeated with four different groups. The average climbing time ($\pm$ s.d.) was calculated for each genotype. Flight assay was performed as previously described¹¹ with 3-day-old males ($n > 100$).

Quantification of wing and thorax phenotypes. For quantification, the percentage of defective thorax and wing phenotypes of 3-day-old males was measured ($n > 300$).

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LETTERS

Drosophila pink1 is required for mitochondrial function and interacts genetically with *parkin*

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Parkinson's disease is the second most common neurodegenerative disorder and is characterized by the degeneration of dopaminergic neurons in the substantia nigra. Mitochondrial dysfunction has been implicated as an important trigger for Parkinson's disease-like pathogenesis because exposure to environmental mitochondrial toxins leads to Parkinson's disease-like pathology¹. Recently, multiple genes mediating familial forms of Parkinson's disease have been identified, including *PTEN-induced kinase 1* (*PINK1*; *PARK6*) and *parkin* (*PARK2*), which are also associated with sporadic forms of Parkinson's disease^{2–6}. *PINK1* encodes a putative serine/threonine kinase with a mitochondrial targeting sequence². So far, no *in vivo* studies have been reported for *pink1* in any model system. Here we show that removal of *Drosophila PINK1* homologue (CG4523; hereafter called *pink1*) function results in male sterility, apoptotic muscle degeneration, defects in mitochondrial morphology and increased sensitivity to multiple stresses including oxidative stress. *Pink1* localizes to mitochondria, and mitochondrial cristae are fragmented in *pink1* mutants. Expression of human *PINK1* in the *Drosophila* testes restores male fertility and normal mitochondrial morphology in a portion of *pink1* mutants, demonstrating functional conservation between human and *Drosophila* *Pink1*. Loss of *Drosophila parkin* shows phenotypes similar to loss of *pink1* function^{7,8}. Notably, overexpression of *parkin* rescues the male sterility and mitochondrial morphology defects of *pink1* mutants, whereas double mutants removing both *pink1* and *parkin* function show muscle phenotypes identical to those observed in either mutant alone. These observations suggest that *pink1* and *parkin* function, at least in part, in the same pathway, with *pink1* functioning upstream of *parkin*. The role of the *pink1*–*parkin* pathway in regulating mitochondrial function underscores the importance of mitochondrial dysfunction as a central mechanism of Parkinson's disease pathogenesis.

Drosophila melanogaster contains a single *PINK1* homologue (CG4523). As with human *PINK1*, *Drosophila* *Pink1* has a predicted amino-terminal mitochondrial targeting sequence and a serine/threonine kinase domain that shares 43% amino acid identity and 60% similarity with human *PINK1* (Supplementary Fig. S1a). More than 25 *PINK1* missense and truncating mutations have been found in Parkinson's disease patients^{1,4,6}. Many of the residues altered by missense mutations⁴ are conserved in *Drosophila* *Pink1* (Supplementary Fig. S1a).

pink1 messenger RNA was detectable at all developmental stages with the highest expression levels in the adult head and testes (Supplementary Fig. S1b). We generated two chromosomal deletions; both showed very similar if not identical phenotypes and

are probably null alleles (Fig. 1a and Supplementary Fig. S1c). Both *pink1* deletion strains were viable; however, they were completely male sterile and almost completely female sterile. Because proper mitochondrial function is required for spermatogenesis, we examined the *pink1* mutant testes for mitochondrial defects. During spermatogenesis, stem-cell differentiation is followed by mitosis and meiosis with incomplete cytokinesis, creating syncytial cysts of 64 spermatids⁹. Subsequently, mitochondria exhibit marked morphological changes. Specifically, early spermatids undergo mitochondrial aggregation and fusion, creating two giant mitochondria that form a spherical structure known as the nebenkern⁹. Under phase contrast microscopy, such 'onion stage' spermatids can be identified as having two adjacent spherical structures: the nucleus and the nebenkern (Fig. 1b). During spermatid elongation, the nebenkern unfurls to yield two mitochondrial derivatives⁹ (Fig. 1i). In *pink1* mutant males, spermatid nuclei appeared normal; however, nebenkerns were vacuolated (Fig. 1d, g) and this vacuolation persisted during subsequent spermatid elongation (Fig. 1k).

To analyse further these mitochondrial defects, we examined *pink1* mutant testes using transmission electron microscopy (TEM). After elongation, spermatids undergo a process known as individualization, in which the cytoplasmic bridges that link the 64 spermatids within a cyst are broken and excess cytoplasm is extruded⁹. After individualization, each spermatid consists largely of the central axoneme, a microtubule-based structure required for motility, and the mitochondrial derivatives, located next to the axoneme (Fig. 1m). Spermatids in *pink1* mutant testes underwent elongation (Fig. 1k); however, numerous defects in individualization were observed (Fig. 1o). Although the number of spermatids in each cyst, the shape and the size of the axoneme, and the spatial relationship between the axoneme and mitochondria were unchanged (Fig. 1o), the overall architecture of *pink1* mutant cysts was disorganized, and the mitochondria were of variable size and were frequently smaller than those in wild-type cysts (Fig. 1, compare panels o and n). *pink1* mutant cysts also frequently contained a large mass of electron-dense material, which may represent aberrant mitochondria (Fig. 1o).

To ensure that the mitochondrial and individualization phenotypes were indeed due to lack of *pink1* function, we generated multiple lines bearing a *pink1* genomic rescue transgene (Fig. 1a). *pink1*⁵ or *pink1*⁹ males carrying a single copy of this transgene were fertile (98% fertile, *n* = 40). Moreover, defects in mitochondrial morphology and individualization were almost completely suppressed (Fig. 1e, h, l, p). Thus, the male sterility phenotype is due to lack of *pink1* function.

To determine the subcellular localization of *Pink1*, we generated a carboxy-terminal *myc*-tagged version of the *pink1* genomic rescue

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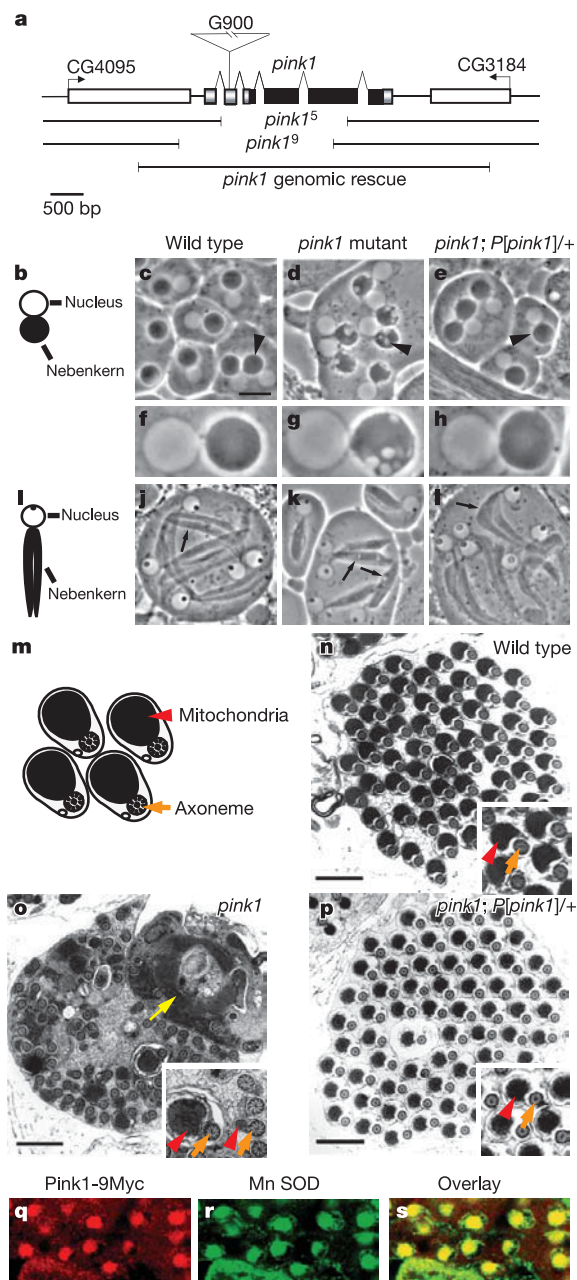


Figure 1 | *pink1* mutants exhibit mitochondrial and individualization defects in spermatids. **a**, Genomic map of *pink1* (cytological location 6C6). P element insertion (triangle), *pink1* coding and untranslated regions (dark and shaded rectangles), and nearby genes (open rectangles) are depicted. The *pink1*⁵ deletion removes the mitochondrial targeting sequence and over 70% of the kinase domain, including motifs required for ATP binding, catalysis and metal binding³⁰. The *pink1*⁹ deletion removes the entire 5' untranslated region (UTR) and part of the kinase domain. The *pink1* genomic rescue construct does not include the full coding region of nearby genes. **b-l**, Schematics and phase contrast micrographs of mitochondrial morphogenesis in spermatids during the 'onion stage' (**b-h**) and spermatid elongation (**i-l**). In both stages, *pink1* mutants show vacuolation of mitochondria (arrowheads and arrows). **m-p**, Schematic and TEM images of single post-individualization cysts containing 64 spermatids. Each spermatid contains an axoneme (orange arrow) and mitochondrial derivative (red arrowhead) within an individual plasma membrane. The *pink1* mutant cyst (**o**) shows individualization defects, mitochondria of variable sizes, and a mass of electron-dense material (yellow arrow). **q-s**, Double labelling of *pink1-9myc* testes with anti-Myc (**q**) and anti-Mn SOD (**r**) demonstrates that Pink1 is localized to the nebenkerns. Scale bars: 10 μ m (**c-e**, **j-l**), 4 μ m (**f-h**) and 1 μ m (**n-p**).

transgene. *pink1* mutant males with the *pink1-9myc* transgene were fertile (100%, $n = 30$), suggesting that the Myc tag does not interfere with Pink1 function. Immunofluorescence on *pink1-9myc* testes demonstrated that Pink1-9Myc protein co-localized to nebenkerns with manganese superoxide dismutase (Mn SOD), a mitochondrial marker¹⁰ (Fig. 1q-s). Together, these observations suggest that *pink1* functions in spermatogenesis to regulate mitochondrial morphology and function.

To determine whether *pink1* has a more general role in mitochondrial function, we examined other tissues in *pink1* mutants. We were unable to detect a significant change in the number of dopaminergic neurons between *pink1* mutant and wild-type flies at 50 days old (Supplementary Fig. S2). However, striking phenotypes were observed in muscle, which also has high energy demands requiring robust mitochondrial function. *pink1* mutants have 'held-up' wings (Fig. 2e) and poor flight performance (Supplementary Fig. S3), suggesting a defect in indirect flight muscles. In wild-type adults, muscle fibres were well organized in parallel stripes with regular banding and no vacuolation (Fig. 2b). In contrast, muscle fibres from age-matched 14-day-old *pink1* mutants were disorganized with prominent vacuoles (Fig. 2f). Ultrastructural TEM analysis showed that the mitochondrial cristae were fragmented in *pink1* mutants, with some mitochondria appearing nearly hollow (Fig. 2g, h), as compared to wild-type mitochondria, which were filled with densely packed cristae (Fig. 2c, d). All of these phenotypes were fully suppressed by the *pink1* genomic rescue transgene (Fig. 2i-l and Supplementary Fig. S3). Previous immuno-electron microscopy studies have shown that mammalian PINK1 is localized to mitochondrial cristae¹¹.

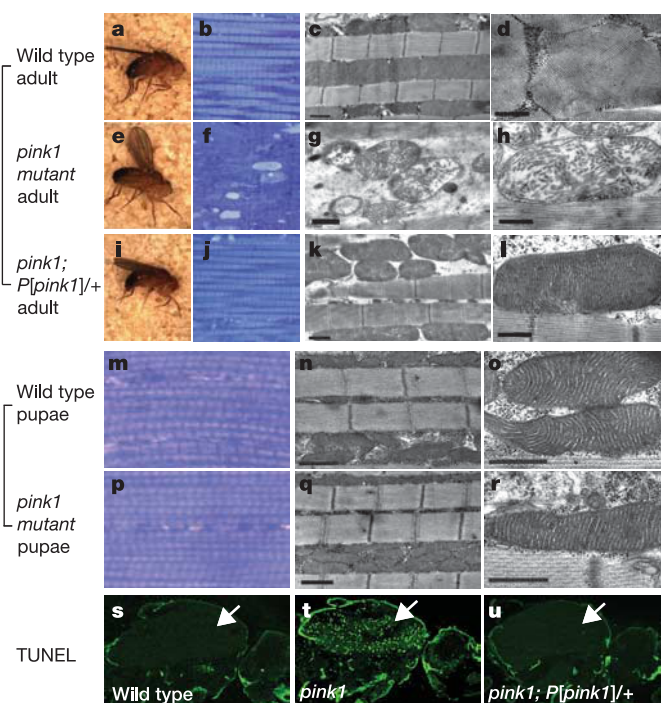


Figure 2 | *pink1* mutants undergo apoptotic muscle degeneration and fragmentation of mitochondrial cristae. *pink1* mutants have held-up wings (compare panels **a** and **e**). **b-d**, **f-h**, **j-l**, Toluidine blue staining (**b**, **f**, **j**) and TEMs (**c**, **d**, **g**, **h**, **k**, **l**) of indirect flight muscle from 14-day-old flies. Muscles of *pink1* mutants show numerous vacuoles (compare **f** and **b**) and mitochondria with fragmented cristae (compare **g**, **h** with **c**, **d**). **m-r**, At 96 h after puparium formation, *pink1* mutant muscle fibres and mitochondria appear normal (compare **p-r** and **m-o**). *pink1*⁵ mutants show many TUNEL-positive nuclei in indirect flight muscle (compare **t** and **s**). Each of these mutant phenotypes is rescued by the *pink1* genomic rescue transgene (**i-l**, **u**). Scale bars: 1.0 μ m (**c**, **g**, **k**, **n**, **q**) and 0.5 μ m (**d**, **h**, **l**, **o**, **r**).

Together, these observations suggest that mitochondrial cristae are a site of Pink1 action, either directly or indirectly.

To investigate whether these muscle phenotypes are developmental or degenerative, we examined indirect flight muscles in *pink1* mutants during development and shortly after eclosion. At 96 h after puparium formation, muscle and mitochondrial morphology of *pink1* mutants (Fig. 2p–r) was indistinguishable from that of age-matched wild-type flies (Fig. 2m–o). At 1–2 days after eclosion, however, *pink1* mutants already showed muscle degeneration and fragmentation of mitochondrial cristae (see below), which was completely suppressed by the *pink1* genomic rescue transgene (data not shown). Furthermore, indirect flight muscles from *pink1* mutants 1–2 days after eclosion demonstrated a marked increase in TdT-mediated dUTP nick end labelling (TUNEL)-positive nuclei (Fig. 2s, t). Taken together, these findings suggest that muscle phenotypes of *pink1* mutants are rapidly degenerative and apoptotic in nature. Finally, ATP levels in *pink1* mutants were significantly reduced (Fig. 3f), demonstrating that mitochondrial function is also severely affected by loss of *pink1*. Apoptosis and reduced ATP levels in *pink1* mutants were fully suppressed by the *pink1* genomic rescue transgene (Figs 2u and 3f).

Mitochondrial dysfunction can lead to decreased resistance to reactive oxygen species, which has been implicated in Parkinson's disease pathogenesis. Sensitivity to reactive oxygen species has also been observed in flies lacking either *parkin*⁸ or *DJ-1*^{12–15}, another familial Parkinson's disease gene¹⁶. To explore the role of *pink1* in resistance to oxidative stress, we analysed the survival of *pink1* mutants after exposure to paraquat, a free radical inducer, or rotenone, which impairs complex I activity in the mitochondrial respiratory chain. Both agents can induce Parkinson's disease-like

pathology in mammals¹⁷. To avoid variability due to genetic background, we included two control strains: a wild-type line with chromosomal background identical to that in *pink1* mutants (precise excision), and a line of *pink1* mutants with the genomic rescue transgene. *pink1* mutants showed 59% reduced resistance to paraquat, and 71% reduced resistance to rotenone (Fig. 3a, b). *pink1* mutants were also sensitive to a hyperoxic environment, a stress that does not rely on feeding (data not shown). The stress sensitivity of *pink1* mutants was not limited to oxidative stress, however, as they were also sensitive to dithiothreitol (DTT), a protein folding inhibitor, and high concentrations of salt, an osmotic stress (Fig. 3c, d). Finally, *pink1* mutants were shorter lived. At 56 days, only 12% of *pink1* mutants were alive, compared with 70–80% of wild-type flies and *pink1* mutants with the *pink1* genomic rescue transgene (Fig. 3e). The sensitivity to multiple stresses in *pink1* mutants may reflect a generalized sickness or may be a consequence of mitochondrial dysfunction.

Drosophila Pink1 shares significant homology with human PINK1 (Supplementary Fig. S1a). To test functional conservation of the human and *Drosophila* homologues, we investigated whether human PINK1 could rescue fly *pink1* mutant phenotypes. We expressed human PINK1 using the testes-specific $\beta 2$ -tubulin promoter¹⁸ (TMR-human PINK1), which is expressed in developing spermatids beginning just before the onion stage. Fertility was restored in 17% ($n = 93$) of *pink1* mutant males carrying the TMR-human PINK1 transgene and mitochondrial phenotypes were rescued in these fertile males (Fig. 4; compare panels b and e with a and d). Thus, *Drosophila* and human Pink1 share at least some functional conservation.

parkin mutants show phenotypes similar to *pink1* mutants: male sterility, apoptotic muscle degeneration, fragmentation of mitochondrial cristae and sensitivity to multiple stresses including oxidative stress^{7,8}. We explored whether *pink1* and *parkin* function in the same genetic pathway. First, we determined whether *parkin* overexpression

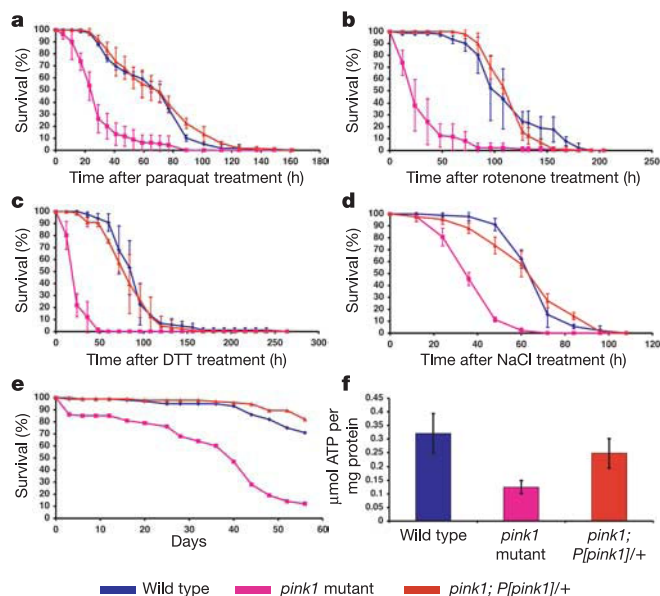


Figure 3 | *pink1* mutants are sensitive to multiple stresses, and have reduced lifespan and ATP levels. a–d, Survival of *pink1*⁵ mutants (pink), wild type (precise excision, blue) and *pink1*⁵ mutants with the genomic rescue transgene (red) after exposure to 20 mM paraquat (a), 5 mM rotenone (b), 100 mM DTT (c) and 500 mM NaCl (d). Mean survival times (in hours) for *pink1*⁵, wild-type and *pink1*⁵; *P[pink1]*/+ flies, respectively, are: paraquat 29.0 ± 6.8, 65.7 ± 1.0 and 70.8 ± 5.5; rotenone 33.6 ± 11.6, 115 ± 14.3 and 116.2 ± 3.6; DTT 25.8 ± 4, 92.4 ± 12.6 and 84.4 ± 9.8; NaCl 40.5 ± 1.5, 68.8 ± 1.8 and 66.6 ± 1.9. e, *pink1*⁵ mutants have reduced lifespan. f, Mean ATP levels (μmol mg⁻¹ protein) of *pink1*⁵ mutants (0.13 ± 0.02) are significantly reduced, as compared with wild-type (precise excision, 0.32 ± 0.07; $P < 0.02$) and *pink1*⁵ flies with the rescue transgene (0.25 ± 0.05; $P < 0.05$). Error bars indicate standard deviation. Student's *t*-test was used.

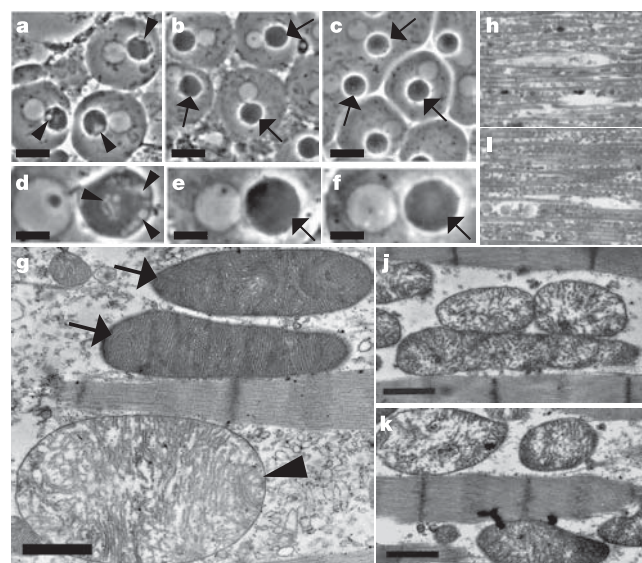


Figure 4 | Fly *pink1* is functionally conserved with human PINK1, and acts upstream of *parkin*. a–f, Onion-stage testes. The vacuolation of nebenkerns (arrowheads) in *pink1*⁵ testes (a, d) is almost completely suppressed (arrows) by the TMR-human PINK1 (b, e) and TMR-*parkin* (c, f) transgenes. g, Overexpression of *parkin* in muscle suppresses the *pink1* mutant phenotype; some mitochondria contain densely packed cristae (arrows), whereas others (arrowhead) still contain fragmented cristae. h–k, Toluidine blue staining (h, i) and TEM (j, k) of 2-day-old adult indirect flight muscles. Double mutants of *pink1* and *parkin* show muscle (i) and mitochondria (k) phenotypes identical to those of *pink1* (h, j) or *parkin* single mutants^{7,8} (data not shown). Scale bars: 10 μm (a–c), 4 μm (d–f) and 1.0 μm (g, j, k).

in *pink1* mutant testes could compensate for loss of *pink1* function and restore male fertility. If *parkin* positively regulates *pink1*, we would expect that overexpression of *parkin* would still result in the *pink1* null phenotype and thus be sterile. Conversely, if *parkin* acts downstream of *pink1*, overexpression of *parkin* might suppress the *pink1* null phenotype and restore fertility. *parkin* overexpression in *pink1* mutants resulted in significant suppression of sterility (62% fertile, $n = 65$) and mitochondrial phenotype (Fig. 4; compare panels c and f with a and d). In contrast, overexpression of *pink1* in the *parkin*^{Δ21} null background⁸ failed to suppress sterility due to lack of *parkin* (0% fertile, $n = 60$). As controls, TMR-*pink1* and TMR-*parkin* restored the fertility in *pink1* and *parkin* mutants, respectively (100% fertile, $n = 40$; 97% fertile, $n = 40$, respectively). Second, when *parkin* was specifically expressed in *pink1* mutant muscle using the UAS/GAL4 system¹⁹, a subset of 1–2-day-old flies contained mitochondria with densely packed cristae (Fig. 4g), which was never seen in age-matched *pink1* mutants (Fig. 4j). When *pink1* was specifically expressed in *pink1* mutant muscle, normal mitochondrial morphology was restored (data not shown). The incomplete rescue in mitochondrial morphology seen with *parkin* overexpression in indirect flight muscles may reflect low expression levels of the transgene. Alternatively, *parkin* overexpression may bypass the requirement for only a subset of *pink1* functions. In either case, these results suggest that *parkin* acts downstream of *pink1*, although it is formally possible that they act in parallel on shared targets.

To test further this hypothesis, we generated double mutants that removed both *pink1* and *parkin* function. If *pink1* and *parkin* act in a linear pathway, double mutants should show phenotypes similar to those observed in either mutant alone. Conversely, if these genes function in parallel pathways to regulate a common process, double mutants would show phenotypes stronger than those of either mutant alone. Double mutants removing both *pink1* and *parkin* function showed mitochondrial and muscle degeneration phenotypes identical to those of either single mutant (Fig. 4h–k and data not shown). Together, these experiments suggest that *parkin* acts, at least in part, downstream of *pink1*. This conclusion is particularly interesting in light of recent work demonstrating that clinical presentations of Parkinson's disease patients harbouring *pink1* or *parkin* mutations are indistinguishable⁶.

Although mild dopaminergic neuronal loss has been observed in *parkin* mutants²⁰, we failed to observe any dopaminergic neuronal loss in *pink1* mutants. Similarly, knockout mice for *parkin* and *DJ-1* generated by multiple groups fail to show any dopaminergic neuronal loss, although functional impairments of the nigrostriatal system and mitochondrial dysfunction have been observed^{21,22}. It is possible that a more sensitive assay, such as mitochondrial morphology or function, or dopaminergic neuronal physiology, may reveal a defect in dopaminergic neurons in *pink1* mutants. However, it is important to note that Parkinson's disease is a multi-system disease affecting more than dopaminergic neurons. Degeneration of many non-dopaminergic neurons including olfactory and brain stem neurons predates that of dopaminergic neurons²³. Moreover, pathological changes and defects in mitochondrial respiratory chain function have been observed in muscle biopsies of Parkinson's disease patients^{24–26}, suggesting more global pathology in Parkinson's disease. Our finding that *pink1* acts upstream of *parkin* to regulate mitochondria strengthens the accumulating evidence that *parkin* has an important role in mitochondrial function, and underscores mitochondrial dysfunction as a central mechanism for Parkinson's disease pathogenesis. The *pink1*–*parkin* pathway provides an entry point for isolating other genes related to regulation of mitochondria, which may include components that function in Parkinson's disease pathogenesis.

METHODS

Molecular biology. To express genes specifically in testes during spermatid differentiation, the pGMR vector²⁷ was modified to replace the glass-binding

sites with part of the regulatory region of $\beta 2$ -tubulin¹⁸, yielding pTMR ($\beta 2$ -tubulin mediated expression).

Genetics and *Drosophila* strains. *pink1* deletions were generated by imprecise excision of the G900 P element obtained from GenExel, which is viable and fertile. Breakpoints were mapped by genomic polymerase chain reaction (PCR) followed by sequencing. For muscle rescue experiments, UAS-*pink1* or UAS-*parkin* was driven by 24B-GAL4 in the *pink1*⁵ mutant background.

Immunofluorescence and confocal microscopy. We used the following antibodies: mouse anti-Myc (Upstate, 1:400), rabbit anti-Mn SOD (Stressgen, 1:300), mouse anti-tyrosine hydroxylase (Immunostar, 1:300).

Light and electron microscopy. For light and electron microscopic analyses, testes were prepared as described previously (refs 9 and 28, respectively). For muscle TEM, thoraces were fixed in paraformaldehyde/glutaraldehyde, post-fixed in osmium tetroxide, dehydrated and embedded in Epon. One-micrometre sections were stained with Toluidine blue, and 50–80-nm sections with uranyl acetate and lead citrate. At least three testes or thoraces of each genotype were examined by TEM.

Stress, flight and longevity assays. 0–3-day-old males were anaesthetized on ice, aged for 48 h, starved for 6 h and subjected to 5% sucrose plus each agent. Three vials of 30–40 flies were assayed simultaneously for each genotype. Flight assays were performed in three groups of 40 3–4-day-old flies, as described previously²⁹. For longevity measurements, 100 males of each genotype were divided into five vials. Flies were maintained at 25 °C and transferred to fresh food every 3–4 days.

ATP and TUNEL assays. For each genotype, ATP levels were determined from lysates of three groups of five 2–4-day-old flies using the ATP bioluminescence assay kit HS II from Roche. Values were normalized to protein content measured by the Bio-Rad protein assay reagent. TUNEL assays were carried out on cryostat sections using the *in situ* cell death detection kit from Roche.

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Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch

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Riboswitches are metabolite-sensing RNAs, typically located in the non-coding portions of messenger RNAs, that control the synthesis of metabolite-related proteins^{1–3}. Here we describe a 2.05 Å crystal structure of a riboswitch domain from the *Escherichia coli* *thiM* mRNA⁴ that responds to the coenzyme thiamine pyrophosphate (TPP). TPP is an active form of vitamin B₁, an essential participant in many protein-catalysed reactions⁵. Organisms from all three domains of life^{6–9}, including bacteria, plants and fungi, use TPP-sensing riboswitches to control genes responsible for importing or synthesizing thiamine and its phosphorylated derivatives, making this riboswitch class the most widely distributed member of the metabolite-sensing RNA regulatory system. The structure reveals a complex folded RNA in which one subdomain forms an intercalation pocket for the 4-amino-5-hydroxymethyl-2-methylpyrimidine moiety of TPP, whereas another subdomain forms a wider pocket that uses bivalent metal ions and water molecules to make bridging contacts to the pyrophosphate moiety of the ligand. The two pockets are positioned to function as a molecular measuring device that recognizes TPP in an extended conformation. The central thiazole moiety is not recognized by the RNA, which explains why the antimicrobial compound pyrithiamine pyrophosphate targets this riboswitch and downregulates the expression of thiamine metabolic genes. Both the natural ligand and its drug-like analogue stabilize secondary and tertiary structure elements that are harnessed by the riboswitch to modulate the synthesis of the proteins coded by the mRNA. In addition, this structure provides insight into how folded RNAs can form precision binding pockets that rival those formed by protein genetic factors.

More than 2% of the genes in some species are regulated by riboswitches^{4,10–13}, whose representative classes compete in number with known metabolite-sensing regulatory proteins¹⁴. On metabolite docking, the sensing domain of the riboswitch shows conformational stabilization, which typically alters the base-pairing arrangements in the adjoining expression platform carrying gene-expression signals^{1–3}. Riboswitches have a remarkable affinity for their cognate ligands and can discriminate against even closely related analogues, as shown by biochemical experiments^{1,13,15} and X-ray structures of purine-sensing riboswitches bound to hypoxanthine¹⁶, guanine¹⁷ and adenine¹⁷. TPP and pyrithiamine pyrophosphate (PTPP; Fig. 1a) are chemically more diverse than purines and pose several additional challenges for negatively charged RNA receptors, namely their large size, conformational flexibility and, most importantly, the presence of negatively charged phosphate groups¹⁸.

We crystallized an 80-nucleotide TPP-sensing domain from the *thiM* mRNA⁴. This RNA conforms well with the consensus sequence and secondary structure model for TPP riboswitches that was established by previous phylogenetic^{6–9} and biochemical^{4,6,19} analyses (Supplementary Fig. S1). The structure (Fig. 1b–d, Supplementary

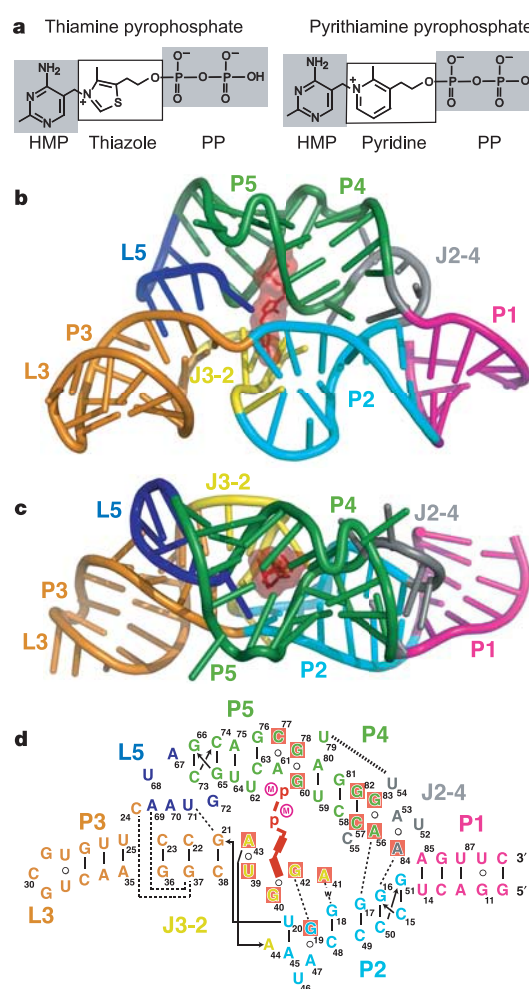


Figure 1 | Structural models of a TPP riboswitch and its ligands.

a, Chemical structures of the natural metabolite TPP and the antimicrobial compound PTPP. **b**, **c**, Crystal structure of the TPP-bound sensing domain, showing front (**b**) and top (**c**) views. The RNA is in a stick-and-ribbon representation, with bound TPP in red. Stems, loops and junctions are colour coded. **d**, Schematic depiction of the RNA tertiary fold observed in the structure. Tertiary contacts formed by hydrogen bonds (w, water-mediated bonds) between bases and stacking interactions are represented by thin and thick dashed lines, respectively. Red shading shows nucleotides conserved in more than 97% of sequences (J. E. Barrick and R.R.B., unpublished observations). Encircled M notations represent Mg²⁺ ions.

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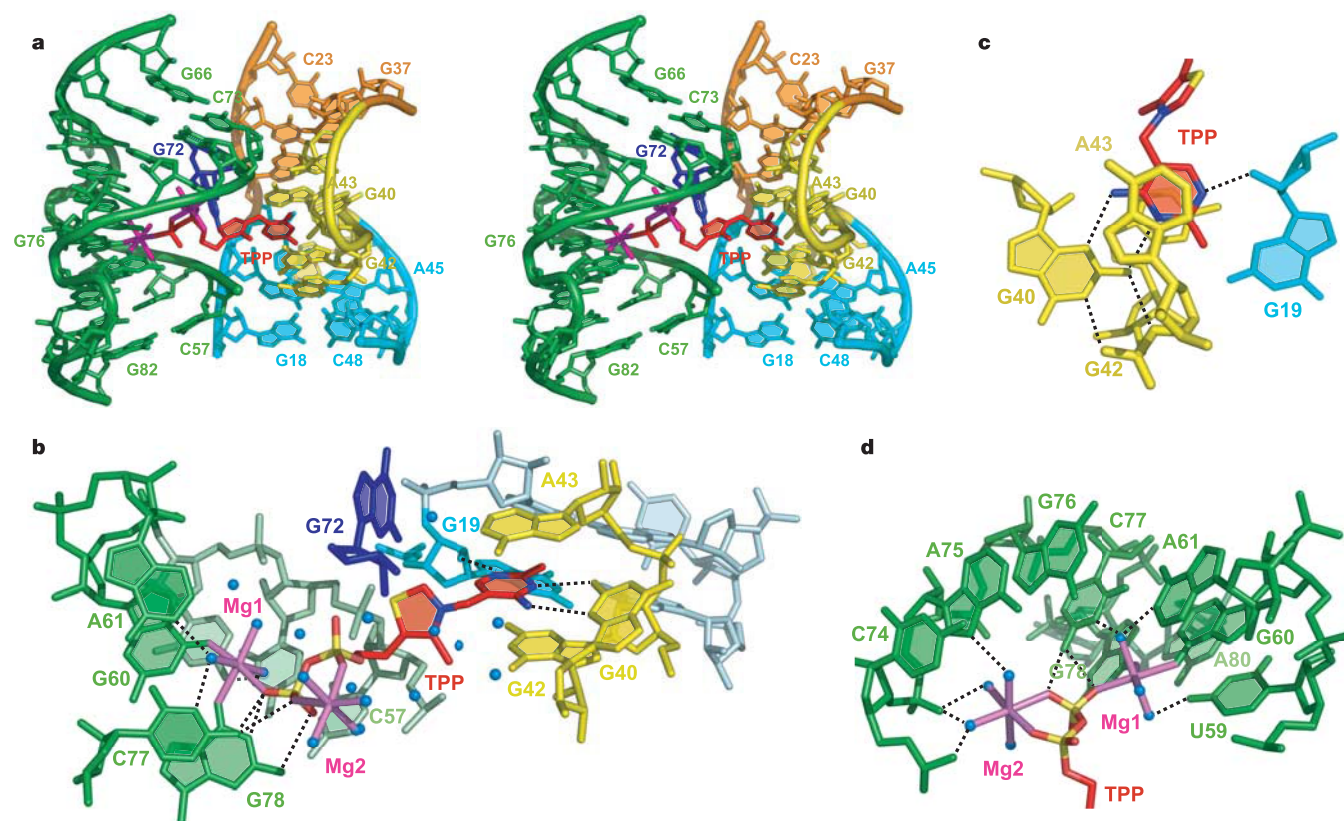


Figure 2 | Structure and interactions in the TPP-binding pocket. **a**, Stereo view of the central region of the complex containing bound TPP. **b**, View of TPP, coordinated Mg²⁺ ions (magenta) and water (blue spheres) in the

binding pocket. **c**, Details of the interactions between the HMP ring and RNA. **d**, Hydrogen bonding between Mg²⁺ ions and RNA.

Fig. S2 and Supplementary Table S1) reveals that the conserved nucleotides and secondary structure elements common to all TPP riboswitches form a complex tertiary architecture consisting of two parallel helical domains (P2/J3-2/P3/L3 and J2-4/P4/P5/L5) connected to a helix (P1) by means of a three-way junction.

Unlike most other ligand–RNA interactions that exploit the helical grooves of RNA, TPP is positioned perpendicular to the two main helical domains and uses separate pockets formed by each helical segment to grip the ends of the compound in an extended conformation (Fig. 2). In addition to the interdomain bridge formed by the ligand, the riboswitch uses tertiary contacts between L5 and P3, and between J2-4, P2 and P4, to assist in stabilizing the global fold.

The J3-2 region (Fig. 1d) is essential in the recognition of the 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) ring. The ring intercalates between G42 and A43, and forms hydrogen bonds between its polar functionalities, previously shown to be critical for molecular recognition⁴, and the 2-OH' of G19 (Fig. 2b, c). This binding arrangement is held by a T-loop-like turn²⁰ formed by the conserved U39–G40–A41–G42–A43 segment, closed by a reverse Hoogsteen U39–A43 pair (Fig. 3a). The backbone is extended at both G40–A41 and G42–A43, resulting in the mutual intercalation and insertion of the HMP ring between these interacting segments. Further, the fold is stabilized by continuous stacking of A41, G42, HMP, A43 and G21, and by the formation of (G19–A47)–G42 and (G18–C48)–A41 triples (Fig. 2a and Supplementary Fig. S3).

TPP riboswitches were the first of several classes found to make productive interactions with negatively charged phosphate groups^{4,12,15}. The pyrophosphate group of TPP is bound in a spacious pocket by a pair of hexa-coordinated Mg²⁺ ions (Mg1 and Mg2) with octahedral ligation geometry that are positioned by direct and water-mediated hydrogen bonds with RNA (Fig. 2d and Supplementary Fig. S1). The terminal phosphate of TPP is coordinated to both Mg1

and Mg2, whereas the thiazole-linked phosphate is coordinated to Mg2. Mg1 is directly coordinated to the O⁶ carbonyls of the conserved G60 and G78 that are located in the region previously implicated in pyrophosphate recognition⁴ (Fig. 2b, d, and Supplementary Fig. S2). In addition, the conserved C77 and G78 form hydrogen bonds to the oxygen atoms of the terminal phosphate of TPP.

An analysis of 72 structures of proteins bound to TPP or its analogues from the Protein Data Bank indicates that proteins typically use charged amino acids to position Mg²⁺, Ca²⁺ or Mn²⁺ ions in a site equivalent to Mg2. However, the Mg1 ion is unique to TPP riboswitches. A bivalent cation at this location allows TPP to reach into the pyrophosphate-binding pocket, thereby stabilizing tertiary interactions important for gene regulation and corroborating the requirement of Mg²⁺ for TPP binding in eukaryotic²¹ and bacterial (Supplementary Fig. S4) TPP riboswitches. One could consider the ligand as TPP with two bound Mg²⁺ ions, which overcomes the ligand's strongly negative electrostatic character. Other riboswitches could use similar simple structural arrangements in combination with cations and water to bind phosphorylated ligands selectively, which would make RNA surprisingly adept at binding negatively charged ligands. The dual bivalent cation arrangement is also distinct from that found in ribosomal RNAs that bind non-phosphorylated antibiotics, in which some contacts with RNA are mediated by single cations^{22–24}.

Nucleotides distal to the ligand-binding sites, conserved in sequence or secondary structure, are important in forming the overall architecture of the TPP-sensing domain. J2-4 (Fig. 1d) is stabilized through the formation of a pair of stacked tetrads, where highly conserved A56–G83 and A53–A84 non-canonical pairs (Supplementary Fig. S5a, b) are aligned in the minor groove of adjacent G–C pairs within P2, forming type I A-minor motifs²⁵. Continuous stacking is observed between P1 and P2, and between non-canonical

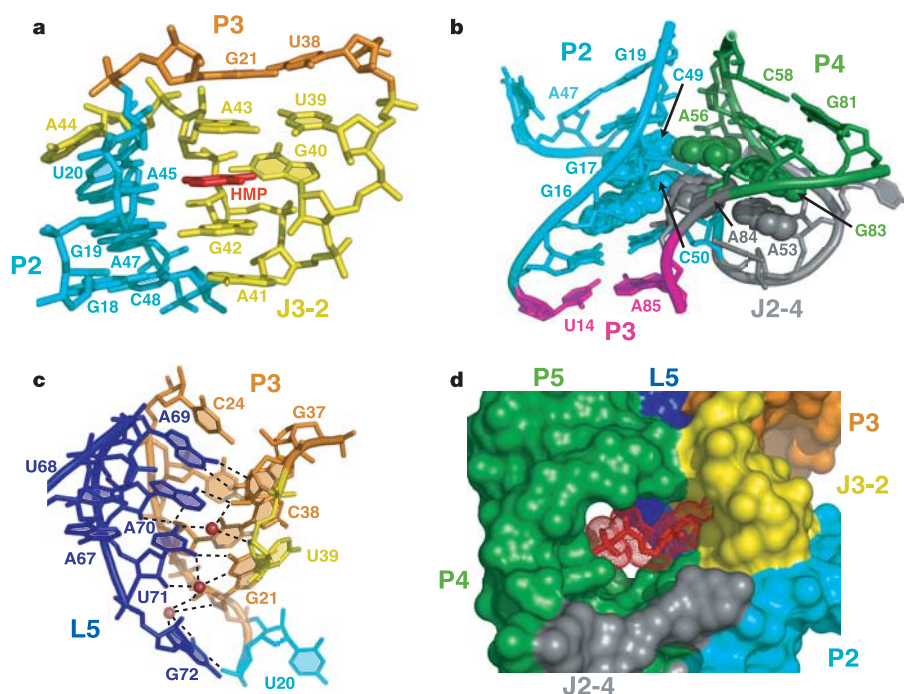


Figure 3 | Tertiary interactions defining TPP riboswitch structure and accessibility to the binding pocket. a, Interaction between J3/2 and P2, mediated by the HMP ring. **b**, Stabilization of the J2-4 junction by two stacked tetrads (in space-filling representation). **c**, Interactions between L5

and P3 mediated by three K^+ ions (red spheres). **d**, Surface representation of RNA and accessibility to the TPP-binding pocket. TPP is depicted in a stick and mesh representation.

pairs in J2-4 and P4 (Fig. 3b). This structural arrangement is expected to be stabilized by ligand binding, and reinforces previous suggestions^{4,12,13} that stabilization of P1 stems in riboswitches is important for the control of gene expression.

Another key tertiary interface involves multiple base–base and base–backbone interactions between nucleotides of L5 and P3 (Fig. 3c), thereby locking into register side-by-side arrangements of P4/P5 and P2/J3-2/P3 segments (Fig. 1b). Three adjacent K^+ ions additionally stabilize the interactions (Fig. 3c). It should be noted that nucleotides of the L5/P3 interface are poorly conserved, and other TPP riboswitches could be stabilized by alternative tertiary contacts spanning this region.

A surface view of the complex shows that the thiazole and diphosphate moieties of TPP are visible, whereas the intercalated HMP ring is buried (Fig. 3d). The deep insertion of the HMP ring most probably implies a conformational transition within the riboswitch upon binding TPP. Support for this interpretation comes from in-line probing experiments, which have identified numerous internucleotide linkages that become conformationally restricted on TPP binding⁴. The structure shows that these regions either contact the ligand directly or participate in critical contacts that form the global structure of the sensing domain.

To assess global conformational changes brought about by ligand binding, we conducted nuclease V1 (helix-specific) and nuclease T2 (single-strand-specific) partial digests of the free and ligand-bound riboswitches. In the absence of TPP these nucleases cleave regions that participate in TPP binding, such as nucleotides (nt) 39–44 (J3-2) and nt 59–61 (part of P4/P5), as well as some regions that are not directly involved in formation of the TPP-binding pocket, such as nt 67–72 (L2) and nt 84–86 (Fig. 4 and Supplementary Figs S6 and S7). The addition of TPP decreases nuclease cleavage in regions that are important for both ligand binding and RNA folding (Fig. 4a, c). These results indicate that TPP not only stabilizes the two sections of the binding pocket by bridging the J3-2 and P4/P5 regions but also promotes the formation and/or stabilization of distal tertiary contacts within the J2-4/P2/P4 region and between L5 and P3.

Tertiary contacts associated with the TPP-stabilized fold of the riboswitch are also apparent in primer extension assays using reverse transcriptase (RT). In the presence of various analogues, including thiamine monophosphate (TMP), RT does not alter its pattern of pausing compared with that observed in the absence of ligand (Fig. 4b). This probably reflects the inability of TMP to span the two ligand-binding portions of the sensing domain fully and to stabilize the global fold, consistent with the progressive loss of binding affinity for thiamine ligands that carry one or no phosphates⁴. However, in the presence of TPP, RT pauses at two distinct positions (Fig. 4b). One pause at G86 occurs close to a pair of stacked tetrads anchoring tertiary contacts involving J2-4, P2 and P4, indicating that this conserved structural element becomes stabilized on ligand binding (Fig. 4c). A second RT pause occurs at C74 adjacent to the tertiary contact between L5 and P3, which seems to be sufficiently strong despite the disruption of base pairing in P1, P4 and P5 during primer extension.

The composite TPP-binding pocket can be defined as a molecular ruler in which only a ligand of proper length can stabilize tertiary interactions and modulate gene expression. This structural characteristic allows the riboswitch to achieve up to about 3,000-fold discrimination against shorter TMP and thiamine, and to provide feedback gene regulation only in the presence of biologically active TPP (ref. 4). In contrast to such unprecedented discrimination of related ligands by the RNA distance ruler, purine riboswitches have developed a completely different approach to distinguishing between related ligands. Adenine-specific and guanine-specific riboswitches use a single tight pocket to position their ligands precisely for Watson–Crick pairing with the discriminatory nucleotide, uracil or cytosine, respectively, thus making a single nucleotide the key element governing riboswitch regulation^{16,17}.

Two predominant modes of TPP-dependent gene regulation, translation inhibition and transcription termination, are illustrated in Fig. 4d. Both require an over-threshold concentration of TPP in the cell for interaction with the riboswitch^{4,9,11,19}. As evident from our biochemical experiments, binding of TPP to the riboswitch induces

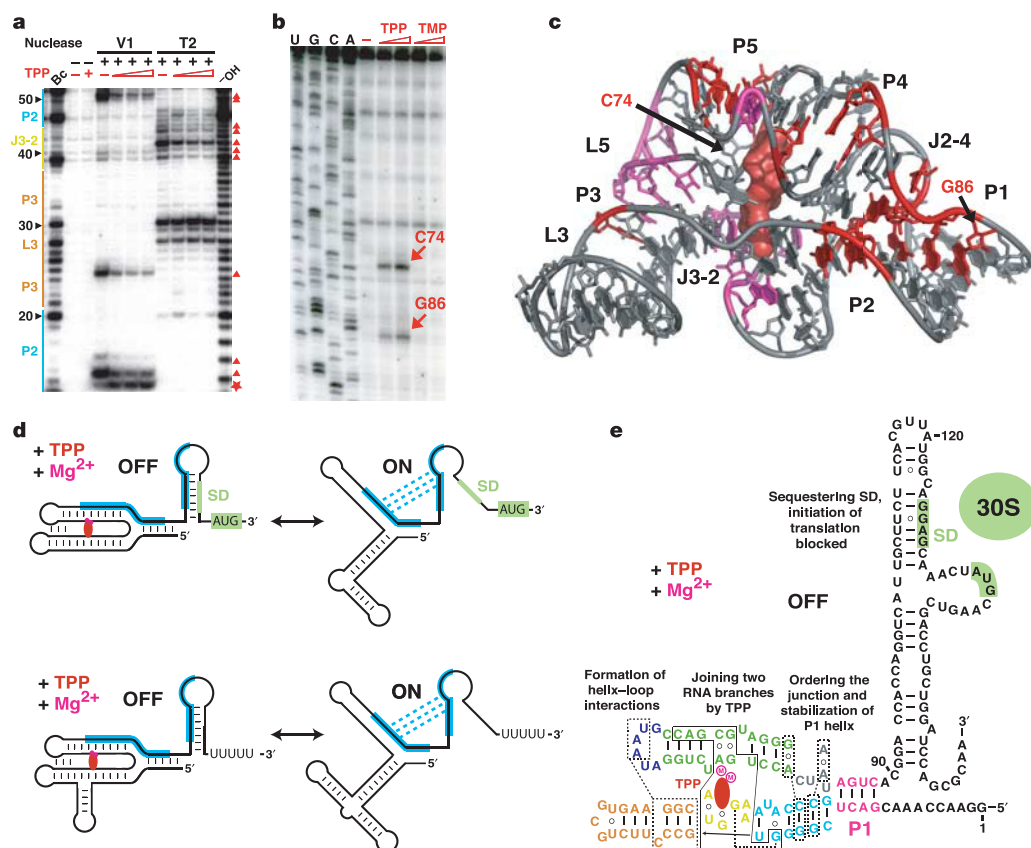


Figure 4 | Structural probing of the TPP riboswitch and implications for TPP-mediated gene repression. **a**, Representative RNase V1 and T2 cleavage patterns for the *thiM* riboswitch (nt 1–166). 5' 32 P-labelled RNAs were treated by nucleases in the absence (–) or presence (+) of one, three and ten equivalents of TPP as described in Supplementary Methods and ref. 30. $^{-}$ OH and Bc stand for ladders prepared by partial digestion with alkali or *B. cereus* RNase, respectively. The major cleavage protections and enhancements in the presence of TPP are labelled with triangles and stars, respectively. **b**, Primer extension analysis in the absence and presence of TPP

or TMP. RT pauses are indicated by arrows. **c**, Summary of structure probing experiments. Major TPP protections against V1 (red) and T2 (magenta) RNases are shown. The RT pauses are indicated by arrows. **d**, Typical mechanisms of TPP-specific gene repression. Top: translation initiation regulation (*thiM* genes). Bottom: transcription termination regulation (*thiC* genes). Complementary sequences and alternate base-pairing are shown in blue. SD sequence and initiation codon are shaded green. TPP and Mg^{2+} ions are depicted in red and magenta, respectively. **e**, Diagram of the OFF state of the *E. coli thiM* riboswitch.

conformational rearrangements leading to stabilization of the overall RNA fold and, most importantly, to stabilization of conserved tertiary contacts adjacent to P1, as defined in the crystal structure (Fig. 4e). These interactions, in turn, stabilize P1 and promote folding of the expression platform, either to a hairpin that sequesters the Shine–Dalgarno (SD) sequence or to a terminator hairpin (OFF state); this results in the failure of translation initiation or premature transcription termination, respectively (Fig. 4d). Without TPP the riboswitch adopts alternative conformations, forming an anti-terminator hairpin or opening the SD sequence for ribosome binding (ON state).

Recent research¹⁹ has revealed that the antimicrobial compound PTPP (Fig. 1a) binds to bacterial and fungal TPP riboswitches and can turn off the expression of critical biosynthetic genes. The structure indicates that the loss of PTPP activity, associated with drug-resisting mutations in TPP riboswitches, might be due to the disruption of key tertiary contacts made by tetrads (C50G, A84G, G86A) and J3-2 elements (A47Δ) (Fig. 3a, b). PTPP carries a pyridine ring in place of the thiazole ring of TPP (Fig. 1a). Because the TPP riboswitch does not make any substantive contacts with the ligand in this chemically distinctive region, our structure-based model reveals why PTPP functions as a mimic of the natural ligand (Supplementary Fig. S8). Given the important functional role of riboswitches in numerous microorganisms and the fact that riboswitches have not yet been detected in the human genome, structures of TPP and other riboswitch classes should enable researchers to employ rational drug discovery strategies to create

novel classes of antibacterial and antifungal compounds that target riboswitches^{19,26,27}.

METHODS

Crystallization. The TPP–riboswitch complex was prepared by mixing RNA transcribed *in vitro* and TPP in a buffer containing 50 mM potassium acetate pH 6.9 and 5 mM $MgCl_2$. Crystals were grown by hanging-drop vapour diffusion. The complex solution and reservoir solution (28% w/v poly(ethylene glycol) 4000, 100 mM sodium acetate pH 4.8 and 200 mM ammonium acetate) were mixed in a 1:1 ratio and incubated at 20 °C. For soaking, crystals were washed with stabilizing solution lacking $MgCl_2$ and then incubated in the presence of 3 mM $Os(NH_3)_6$ for two days.

Structure determination. Native and $Os(NH_3)_6$ multiple anomalous diffraction (MAD) data were collected at beamline X25 at the Brookhaven National Synchrotron Light Source. Data were processed with the HKL2000 suite of programs (HKL Research). The structure was determined by using MAD osmium data and SOLVE/RESOLVE²⁸. The RNA model was built using TURBO-FRODO (<http://afmb.cnrs-mrs.fr/rubrique113.html>) and refined with REFMAC²⁹ using a native data set (Supplementary Figs S9–S11). TPP and cations were added to the model on the basis of analysis of $2F_o - F_c$ and $F_o - F_c$ electron density maps. Na^+ , K^+ and hydrated Mg^{2+} were modelled on the basis of the number of coordination bonds, their distances and their coordination geometry. RNA residue 55 has a partial electron density.

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Author Information Coordinates of the X-ray structure of the TPP-bound riboswitch have been deposited in the RCSB Protein Data Bank under accession code 2GDI. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.S. (serganoa@mskcc.org) or D.J.P. (pateld@mskcc.org).

LETTERS

Structure of the *S*-adenosylmethionine riboswitch regulatory mRNA element

Rebecca K. Montange¹ & Robert T. Batey¹

Riboswitches are *cis*-acting genetic regulatory elements found in the 5'-untranslated regions of messenger RNAs that control gene expression through their ability to bind small molecule metabolites directly^{1,2}. Regulation occurs through the interplay of two domains of the RNA: an aptamer domain that responds to intracellular metabolite concentrations and an expression platform that uses two mutually exclusive secondary structures to direct a decision-making process. In Gram-positive bacteria such as *Bacillus* species, riboswitches control the expression of more than 2% of all genes through their ability to respond to a diverse set of metabolites including amino acids, nucleobases and protein cofactors^{1,2}. Here we report the 2.9-Å resolution crystal structure of an *S*-adenosylmethionine (SAM)-responsive riboswitch from *Thermoanaerobacter tengcongensis* complexed with *S*-adenosylmethionine, an RNA element that controls the expression of several genes involved in sulphur and methionine metabolism^{3–6}. This RNA folds into a complex three-dimensional architecture that recognizes almost every functional group of the ligand through a combination of direct and indirect readout mechanisms. Ligand binding induces the formation of a series of tertiary interactions with one of the helices, serving as a communication link between the aptamer and expression platform domains.

Genetic regulation by the *S*-adenosylmethionine (SAM) riboswitch is driven through the binding of SAM to the aptamer domain, which consists of a series of stem and stem-loop structures centred on a four-way junction motif (Fig. 1a, Supplementary Fig. 1). Among the more than 150 identified SAM-I sequences⁷, several nucleotides within and surrounding the junction are highly conserved phylogenetically, including a consensus kink-turn motif⁸ and a genetically

validated pseudoknot⁹. Stable formation of this domain through its interaction with SAM causes the downstream expression platform to create a rho-independent transcriptional terminator, turning off gene expression (Supplementary Fig. 1). In the absence of sufficient intracellular SAM, the 3' side of the P1 helix forms an antiterminator element, allowing transcription of the mRNA.

To understand the structural basis for the specific recognition of SAM by a riboregulator and how binding is transduced into changes in gene expression, we have solved the structure of the SAM riboswitch RNA at 2.9 Å (Supplementary Table 1 and Supplementary Fig. 2). The global architecture of the SAM-I riboswitch aptamer domain is established through two sets of coaxially stacked helices. The first comprises helices P1 and P4 and the intervening J4/1 joining region (blue in Fig. 1b). J4/1 contains three conserved purine residues (A83–A85) stacked between the two helices, with A85 pairing with U64 of J3/4 and A24 of L2 to anchor helices P1/P4 against the other set of coaxial stacked helices, P2 and P3 (green in Fig. 1b). The two sets of helices pack together at a roughly 70° angle with the ligand-binding pocket situated between the minor grooves of the P1 and P3 helices. This arrangement of the P1/P4 and P2/P3 stack is architecturally similar to the P4–P6 and P3–P7 domains that form the conserved catalytic core of all group I introns^{10–12} (Fig. 2). Each RNA consists of a pair of nearly coaxially stacked helices (P1/P4 and P4–P6; blue in Fig. 2) that serve to scaffold a second domain (P2/P3 and P3–P7; green in Fig. 2) that wraps around the first to complete the active site. The interactions between the two domains is stabilized by minor-groove–minor-groove packing near the point of covalent attachment by single-stranded joining regions along with a loop from the second domain forming tertiary interactions with

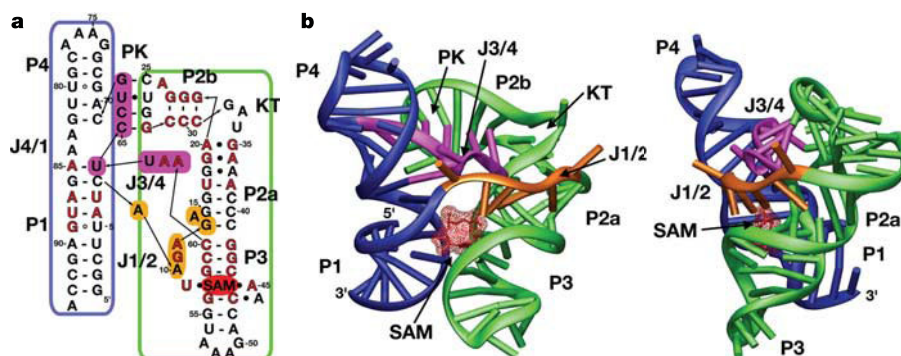


Figure 1 | Secondary and tertiary structure of the SAM-I riboswitch.

a, Secondary structure of the SAM-I riboswitch aptamer domain reflecting the tertiary organization; nucleotides more than 95% conserved across phylogeny are highlighted in red. Solid arrows represent the direction of the RNA backbone, and elements of structure are labelled as follows: P, paired;

J, joining; KT, kink-turn; PK, pseudoknot. Colours used to denote elements of structure are consistent with subsequent figures. **b**, Ribbon representation of the three-dimensional structure. The two views represent a front view (left) and a 90° clockwise rotation (right). SAM is in red with its surface represented as dots.

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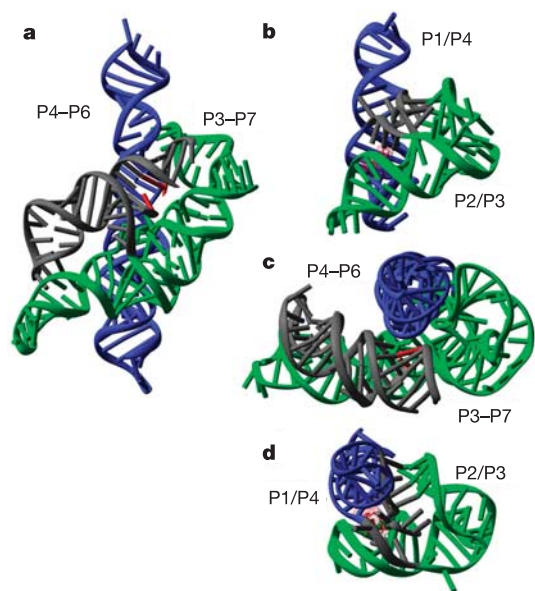


Figure 2 | Comparison of the global architecture of the *Azoarcus* group I intron and the SAM-I riboswitch. **a, c,** Side (**a**) and top (**c**) views of the group I intron with the two principal conserved domains P4–P6 and P3–P7 coloured in blue and green, respectively. The active site is highlighted by the two red nucleotides, which represent the site of cleavage. **b, d,** Side (**b**) and top (**d**) views of the SAM-I riboswitch in the same orientation as the group I intron, with the two primary domains P1/P4 and P2/P3 coloured in blue and green, respectively.

the first (L2 in the SAM riboswitch and a tetraloop in the group I intron).

A ligand-independent set of tertiary interactions between L2, J3/4 and J4/1 establish the global architecture of the RNA. Within helix P2 is a kink-turn motif that creates a roughly 100° bend in the helix; this element is identical in consensus sequence, pattern of non-canonical base pairing and three-dimensional fold to those observed in the ribosome¹³, the Box C/D snoRNP¹⁴ and the U4 snRNP¹⁵, despite the lack of a bound protein. The kink-turn orients helix P2b back towards the P1/P4 stack, permitting a pseudoknot interaction between L2 and J3/4, as predicted from phylogenetic alignments⁶ and genetic experiments⁹. The pseudoknot is tied against P1/P4 through the (A85–U64)•A24 triple (Fig. 3). J3/4 is further tied to helix P2b through two adenine-mediated triples in which the Watson–Crick face of A61 and A62 interact with the minor groove of the G22–C30 and G23–C29 pairs, respectively (Fig. 3a). This form of adenosine triple is not nearly as common as the A-minor triple motif¹⁶ but is observed in the 16S rRNA¹⁷. In-line probing of several different SAM-I riboswitches revealed that key nucleotides in L2, J3/4

and J4/1 involved in the formation of this region of tertiary structure are protected from cleavage in the absence and presence of SAM^{6,18} indicating that these structures are not ligand dependent. The SAM-I riboswitch, similarly to the purine riboswitch^{19,20} and most probably the other riboswitches, has a pre-established architecture comprising the general coaxial stacking of helices and tertiary structural elements outside the ligand-binding pocket that serve to facilitate ligand recognition.

S-adenosylmethionine is bound within a pocket created between the P1 and P3 helices and J1/2 (Fig. 4a). The ligand adopts a compact conformation in which the methionine moiety stacks upon the adenine ring, stabilized in part by a π -cation interaction with the amino group pointing towards the adenine ring (Supplementary Fig. 3). This conformation of SAM is very different from that found in most proteins (Supplementary Fig. 4) in which SAM typically adopts an extended *trans* configuration, with the adenosyl and methionine moieties projecting away from one another²¹. Only a few methyltransferases and the repressor metJ bind SAM in a similarly compact fashion²¹, although none possesses the degree of stacking between the methionine and adenine moieties observed in the RNA-bound form. Calculations of the most favoured conformations of S-adenosylhomocysteine (SAH) show that the RNA-bound configuration of SAM is close to the energetically most favourable form of SAH²², indicating that the RNA probably does not distort the geometry of the ligand on binding.

The compact configuration of SAM creates two distinct faces that interact with the RNA in different fashions, one dominated by hydrogen-bonding interactions to helix P3 and the other using van der Waals surface complementarity with the minor groove of the P1 helix. The adenine ring of SAM is the central base of a triple between A45 and U57 (Fig. 4b). These two nucleotides are part of an asymmetric internal loop motif (5'-AA/U) in helix P3 (Fig. 1a) that is universally conserved among SAM-I riboswitch RNAs. The placement of the adenine ring is further stabilized by stacking with C47, which is part of a dinucleotide platform (A46•C47–G56)^{23,24}. The main chain atoms of the methionine moiety are recognized through a series of hydrogen bonds with the G11•C44–G58 triple between helices P3 and J1/2 (Fig. 4c). The negatively charged carboxylate group is recognized through interactions with the Watson–Crick face of G11 (N1 and N2) in a commonly used mode of binding carboxylate and phosphate groups by RNAs²⁵. Consistent with the structure is the observation that whereas SAH is tightly bound by the RNA ($K_d = 400$ nM in the *yitJ* homologue from *B. subtilis*), the analogue S-adenosylcysteine, containing one less side-chain methylene group, binds significantly more weakly ($K_d \approx 30$ μ M)⁶. Thus, the length of the methionine side chain is sensed through an indirect readout mechanism requiring two methylene groups to place the carboxyl group within hydrogen-bonding distance of G11.

The other side of the binding pocket for SAM is created by the

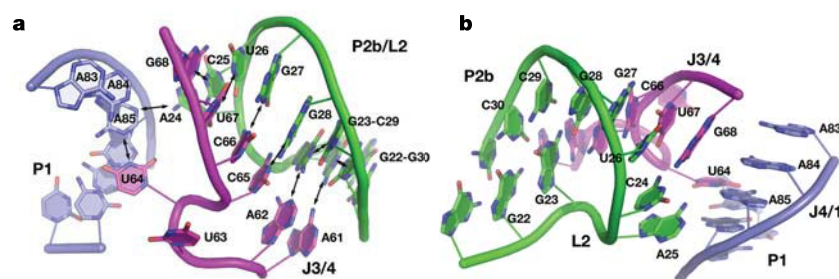


Figure 3 | Detailed view of the tertiary architecture of the pseudoknot region. **a,** Top view relative to Fig. 1c, left. The J3/4 strand ties the P1/P4 stack to the P2/P3 stack by means of base interactions. These comprise A-minor triples mediated by A61 and A62 on the 5' end of J3/4 with helix

P2b, a base triple between U64, A85 and A24, and a pseudoknot between C25–G28 of L2 and C65–G68 of J3/4. **b,** View of this region from the back relative to Fig. 1c, left, emphasizing the pseudoknot interaction and its orientation relative to the P1/P4 stack.

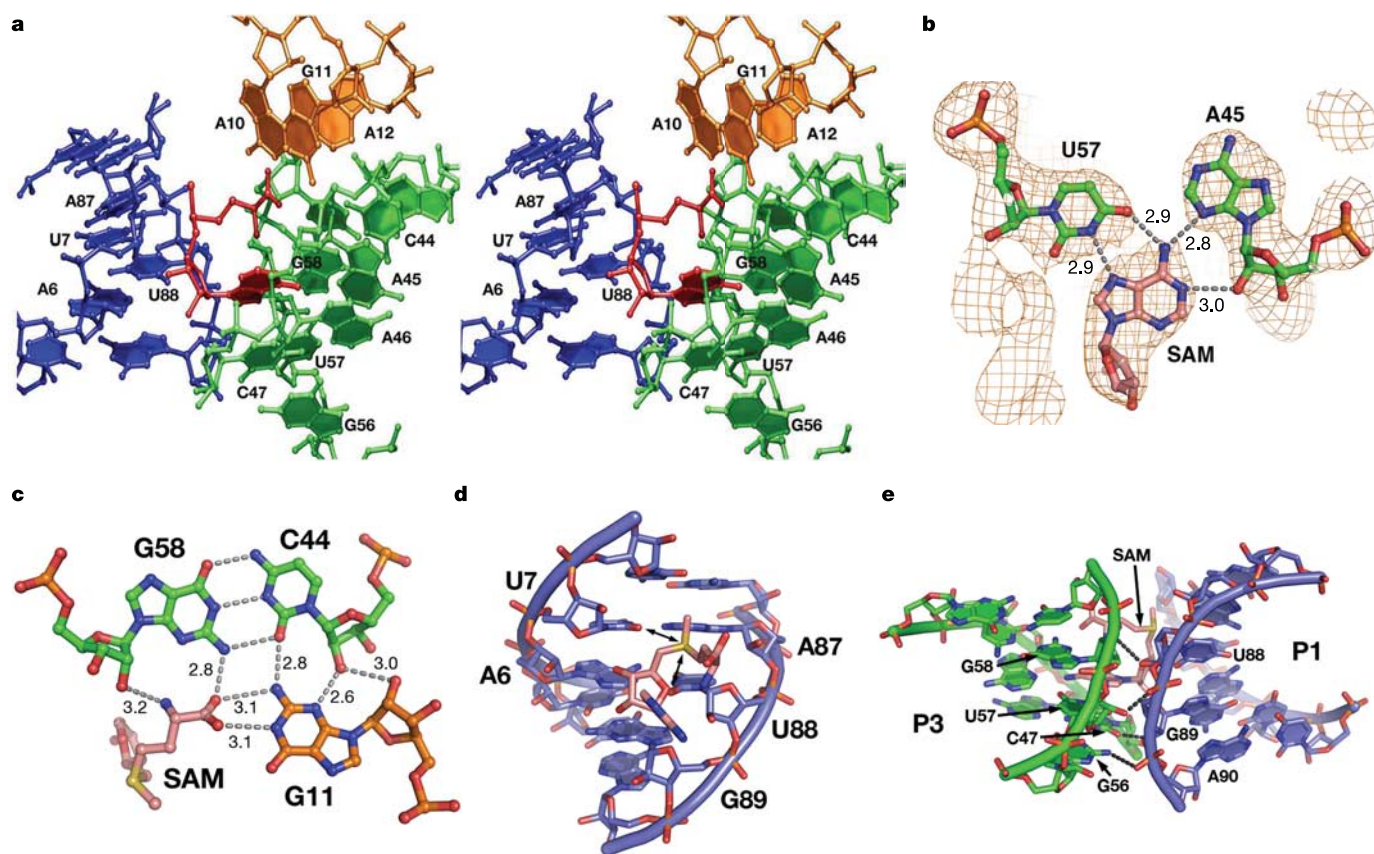


Figure 4 | Detailed view of SAM recognition by the riboswitch. **a**, Stereo view of the SAM-binding pocket comprising the P1 and P3 helices and J1/2. **b**, Hydrogen-bonding interactions between the adenine base of SAM, A45 and U57. The experimental electron density map is shown as an orange cage, contoured at 1.25 σ . **c**, Interactions between the main chain atoms of

methionine and the G58–C44 pair in P3 and G11 of J1/2. Distances in **b** and **c** are given in ångströms. **d**, SAM binding to the P1 helix; selectivity for SAM is probably mediated through electrostatic interactions with the carbonyl O2 of U7 and U88 (double-headed arrows). **e**, Ligand-induced interactions between the P3 helix and the 3' side of the P1 helix (black dashed lines).

minor groove of the P1 helix, adjacent to the universally conserved A6–U88 and U7–A87 base pairs. The ribose sugar of SAM is situated in such a way that it forms a direct hydrogen bond only between its 2'-hydroxyl and O4 of C47 in helix P3. Interactions between SAM and helix P1 seem to be restricted to van der Waals packing with U7 and U88, which is consistent with binding studies with SAM analogues containing deletions of the 2'- and 3'-hydroxyl groups¹⁸. The sulphur atom is situated about 4 Å from the O2 carbonyl oxygens of U7 and U88 (Fig. 4d). This positioning serves as the basis for a 100-fold preference for SAM over SAH^{6,18}; the positively charged sulphur is positioned to make favourable electrostatic interactions with the carbonyls of the minor groove of helix P1. This RNA has evolved to not use the anionic phosphate backbone for the recognition of either of the positively charged functional groups in SAM. Biochemical observations indicate that the identity of the charged moiety at this position is not important, but the presence of a formal positive charge or high partial positive charge is required¹⁸. The methyl group points towards a solvent cavity within the interior of the RNA and is not directly recognized, as previously predicted⁶. This structure is therefore consistent with all of the observed genetic, biochemical and phylogenetic data available for this class of riboswitches.

The mechanism by which the SAM-I riboswitch transduces a binding event into changes in gene expression is revealed by this structure. The interaction between SAM and the mRNA induces a series of tertiary interactions involving hydrogen-bonding interactions between backbone ribose–phosphate groups of U88–A90 in helix P1 and C47, G56–G58 of helix P3 and van der Waals interactions between the ribose sugars of C48 and A90 (Fig. 4e). These

contacts are clearly ligand induced, as revealed by in-line probing, which shows strong protection of these nucleotides only on association with SAM^{6,18}. In addition, the ligand is about 74% inaccessible to solvent (Supplementary Fig. 5); this degree of burial requires that helices P1 and P3 close around the ligand during the binding event, forming the new tertiary interactions.

Similarly to the purine riboswitch^{19,20}, the 3' side of the P1 helix is buttressed through an extensive network of hydrogen-bonding, electrostatic and van der Waals interactions with both the ligand and other regions of the RNA, establishing the basis for gene regulation. In both cases, this side of the P1 helix is an integral part of a structural switch involving two mutually exclusive secondary structures in the expression platform (Supplementary Fig. 1). Stabilization of the P1 helix communicates the liganded state of the aptamer domain to the expression platform by preventing formation of the antiterminator element, causing the expression platform to form a terminator element. Because all of the riboswitches (with the exception of the *glmS* riboswitch-ribozyme²⁶) have a similar secondary structural organization in which a P1-like stem directly links the aptamer domain with the expression platform, this mechanism is probably common to all. Thus, ligand-induced allosteric changes drive effective gene regulation by both RNA-based riboswitches and protein-based transcriptional repressors.

METHODS

Transcription vectors for the synthesis of RNA were constructed and transcribed with the use of techniques described previously²⁷, and purified with 12% denaturing polyacrylamide gels. After purification, the RNA was concentrated, refolded by heating and cooling, and SAM was added to a final concentration of

5 mM. Crystals were grown by mixing this solution in a 1:1 ratio with mother liquor (100 mM KCl, 5 mM MgCl₂, 10% 2-methyl-2,4-pentanediol 10 mM sodium cacodylate pH 7.0, 8 mM iridium hexa-ammine, 6 mM spermine-HCl) and incubated for 48 h at 30 °C. A two-wavelength anomalous diffraction experiment was performed on crystals cryoprotected in mother liquor plus 15% ethylene glycol, and data were collected to 2.8 Å resolution. The data were indexed, integrated and scaled with D*TREK²⁸, and all subsequent calculations and refinement were performed with CNS²⁹ to obtain a model containing all RNA atoms as well as the SAM ligand with final R_{crystal} and R_{free} values of 26.7% and 28.8%, respectively. Additional experimental details and references are given in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The atomic coordinates and structure factors have been deposited in the Protein Data Bank with the accession number 2GIS. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to R.T.B. (robert.batey@colorado.edu).

RETRACTION

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Induction of DNA methylation and gene silencing by short interfering RNAs in human cells

Hiroaki Kawasaki & Kazunari Taira

Nature 431, 211–217 (2004); corrigendum *Nature* 431, 878 (2004)

There are several independent papers^{1–3} supporting the existence of siRNA-mediated suppression of transcription associated with histone methylation and DNA methylation in mammalian cells with targeting of a promoter region, as we described in this Letter. Unfortunately, however, a proper data notebook is not available as evidence to support our findings, which constitutes non-adherence to ethical standards in scientific research. In accordance with the recommendations from the National Institute of Advanced Industrial Science and Technology, K.T. therefore wishes to retract this paper.

H.K. maintains that all the data contained in this Letter are valid and so declines to be a co-signatory of this retraction.

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Divide and conquer

A key element of performing good cell-biology experiments is starting with exactly the right cells. Michael Eisenstein takes a look at the technologies that can make this possible.

What do you get when you cross an ink-jet printer with a Coulter counter? It's not a riddle; scientists at Los Alamos National Laboratory in New Mexico asked the question 40 years ago, and the answer turned out to be the cell sorter.

Los Alamos researcher Mack Fulwyler created a prototype in 1965, which used vibration to generate tiny droplets from a jet of solution containing red blood cells; the individual cells in each droplet could then be subjected to rapid volumetric analysis and sorting. Fulwyler's prototype came to the attention of Stanford University researcher Leonard Herzenberg. "I was working on immunofluorescence in immunology and genetics," he says, "and had realized that there was a need for the means to sort cells according to the molecules they display on their surface."

Herzenberg and his colleagues adapted Fulwyler's design to produce an instrument that could sort cells depending on the presence or absence of molecules identified by fluorescent labels — the first example of fluorescence-activated cell sorting (FACS). Today, FACS has not only aged gracefully but is arguably in its prime, embraced by nearly everybody looking



Leonard Herzenberg devised early cell sorters.

to pluck specific cells out of complex mixtures. Herzenberg's patent was licensed by Becton Dickinson (BD), which developed the first commercial instrument and currently holds the trademark for the term 'FACS'. Early FACS fans include Dutch researcher Gerrit Van den Engh, whose refinements enhanced sorting rates. "I designed a different, digital parallel

post-processing scheme," says Van den Engh. "By digitizing the signal as quickly as possible, we could take the cells in parallel and we could put an error-tracking code on the information, so that we could check whether events were being dealt with properly."

Cytomation, later acquired by Dako in Glostrup, Denmark, made Van den Engh's patent the foundation for MoFlo, the first high-speed commercial sorter. MoFlo pushes the speed envelope, sorting up to 70,000 objects per second — although researchers often use lower rates to optimize recovery and sort accuracy. Dako has also ensured that new expansions and components are suitable for use both with current and older systems. "Our modern upgrades are reverse-compatible, even with MoFlo machines from eight or nine years ago," says Cytomation's founder, George Malachowski.

Meanwhile BD Biosciences, a segment of BD in San Jose, California, recently introduced its most advanced cell sorter to date. The BD FACSria benefits from its small size, being one of the few bench-top systems on the market, and from an alternative approach to pre-sorting analysis. Most sorters use the 'jet-in-air'

L. HERZENBERG

THE GENTLE TOUCH

Sorting individual cells is not a problem, but what if a researcher wants to sort clusters of cells, or even whole embryos?

Union Biometrica of Holliston, Massachusetts, offers a potential solution in the shape of its COPAS family of instruments. These, it says, can work with objects ranging from pancreatic islets up to zebrafish hatchlings. The principle is similar to fluorescence-activated cell sorting (FACS) — objects in solution pass through a specially designed flow-cell, where they are optically profiled according to as many as five different sorting parameters (which can include three fluorescent wavelengths), and specimens of interest are diverted and collected.

But some design adjustments were necessary to protect the integrity of the multicellular objects being sorted. For one thing, rather than using charge-based sorting of droplets, COPAS uses

rapid puffs of air to gently divert 'slices' from the flow stream that contain objects of interest.

COPAS also uses lower flow-rates than FACS, sorting between

100 and 300 objects per second. "We sacrifice speed for gentle flow, which results in increased viability and less destruction of what we're analysing," says Rock

Pulak, Union Biometrica's director of life sciences. "It's still much faster than trying to analyse these same numbers of cell clusters by microscope."

In addition, the lower speeds mean that it is possible to do limited analysis of fluorescence localization within the objects being sorted. Pulak speculates that future instruments may even be able to perform actual imaging during the sorting process.

Sometimes even single cells prefer a lighter touch, and an ongoing collaboration between the company and the Joslin Diabetes Center in Boston has shown that COPAS is also useful for sorting adipocytes and hepatocytes.

"These are larger cells that are very delicate and subject to sensitivities with respect to shear force," says Pulak. "We are finding that our technology is appropriate for these kinds of applications."

UNION BIOMETRICA



Union Biometrica's BioSorter COPAS can sort clusters of cells.

M.E.

approach, in which optical analysis is performed on a rapidly moving stream of fluid, but the BD FACSaria instead uses a sorting flow-cell. "This gives you the much higher sensitivity that you need, but maintains extremely efficient sorting," says marketing director Tony Ward. "And you can sort cells that have lower levels of antigen expression than you might be able to see using a jet-in-air approach."

Several alternative systems are available. Beckman Coulter of Fullerton, California, another early entrant into the field, offers its EPICS ALTRA, an established platform for cell sorting. In 2000, Van den Engh launched a new company, Cytopeia, based in Seattle, Washington, whose inFlux Cell Sorter is based on ideas from his academic work. "It's an open system, so you can have access to all the modules and can configure it freely for whatever experiment you want to do," he says. "We're not competing with the other manufacturers for well-established applications; we work with the 10–20% of researchers who have applications that are not done as well on the other machines." And for researchers working with larger objects, Union Biometrika of Holliston, Massachusetts, offers a FACS-like platform for sorting embryos and multicellular clusters (see 'The gentle touch', page 1179).

Most observers agree that cell sorting has probably reached its speed limit, and some scientists are now looking to expand the breadth of flow cytometric analysis and sorting. Mario Roederer of the US National Institutes of Health (NIH) in Bethesda, Maryland, has been a leader



The BD FACSaria uses a sorting flow-cell.

in this regard, combining fluorescent dyes and quantum dots to perform experiments involving simultaneous analysis of up to 17 different intracellular and cell-surface markers. Many cell biologists have yet to explore these outer limits, but current commercial sorters can typically accommodate optics for analysing a dozen or more fluorescent parameters. As an immunologist examining very specific cell subtypes, Roederer finds this flexibility invaluable: "We're routinely doing 12- or 15-colour flow-cytometry to try to look for important subsets or functions. In the end, I'm hoping we'll be able to reduce it to a 4- or 5-colour assay with the correct combination of markers."

With all the power that these cell-sorting systems offer, there are still problems to be resolved. "Software is the issue that requires the most effort," says Roederer. "We need tools that can automate the discovery or the analysis of subsets of cells that are present in complex data sets." Ward agrees: "The faster you count particles, the more data get generated and the

resulting high degree of data complexity and intersections mean that current approaches to software can be limiting." Both Roederer and Herzenberg have worked to address this, developing two commercially available software packages, FlowJo and FACSxpert, intended to improve the quality of cell-sorting analysis.

Another big factor for many is cost: power and efficiency don't come cheap, and access to high-end machines may be restricted to limited slots in a shared core facility. "I'd like to see cheaper machines that give you five, six or seven colours but that are much less expensive than the mammoth machines," says Herzenberg.

Nevertheless, these instruments receive strong acclaim from users. "I don't want to say it's a way of life," says Roederer, "but it is a way of biology."

Working in bulk

Sometimes, however, all a scientist needs is a way to separate two groups of cells quickly. "FACS can do pretty much everything, but it's expensive," says Steven Woodside, a scientist with StemCell Technologies of Vancouver, British Columbia. "If you want to do more bulk separations, then immunomagnetic separation is a really good option."

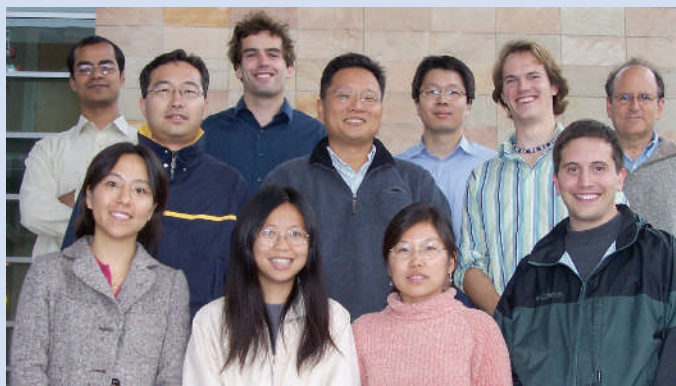
The principle is simple. Cells are incubated with paramagnetic beads tagged with antibodies, after which a magnet or array of magnets can be used to either purify cells of interest or remove unwanted cells. Dynal Biotech's Dynabeads — currently available through Invitrogen in Carlsbad, California — were among the first

BD BIOSCIENCES

PLAYING THE FIELD

Researchers have used electrical fields to manipulate nucleic acids and proteins for more than 50 years, but similar systems have only recently begun to emerge for working with whole cells. Dielectrophoresis is nevertheless quickly gaining appeal as a basis for microfluidic cell-sorting.

Hyongsok (Tom) Soh and Patrick Daugherty at the University of California, Santa Barbara, recently demonstrated the feasibility of dielectrophoresis-activated cell sorting, or 'DACS', with a microfluidic chip that uses dielectrophoretic forces to steer bacteria tagged with polystyrene beads into a collection channel (X. Hu *et al. Proc. Natl Acad. Sci. USA* 102, 15757–15761; 2005). Initial experiments showed that one round of DACS could achieve more than 200-fold enrichment of a rare subpopulation of cells at rates of 10,000 bacterial cells per second. They initially tested a



Tom Soh (middle row, centre) and his team have put together prototype microfluidic chips for dielectrophoretic cell-sorting.

single-stage, single-channel device, but Soh believes DACS is ideal for parallel operations. "It is relatively straightforward to design cascaded, sequential sorting stages that operate in parallel," he says. "This allows high purity and cell recovery without sacrificing throughput."

Soh is quick to point out that DACS is in no position to usurp

fluorescence-activated cell sorting, because of the binary nature of its sorting mechanism; but it shows great promise for high-throughput screening, he says. "We just completed screening the first molecular library and performed epitope mapping with DACS," Soh explains, "and we've shown that it can be faster, cheaper and simpler

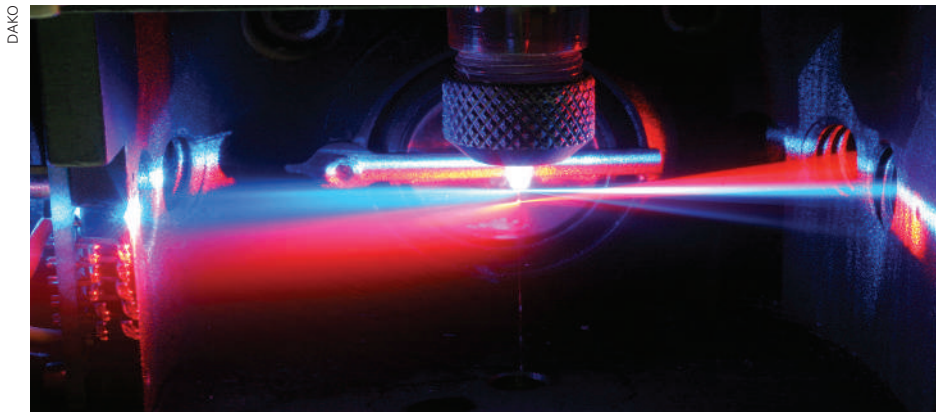
than commercial assays."

Evotec Technologies in Hamburg, Germany, is also taking advantage of dielectrophoresis for its Cytocon 400 system. The key to this is the CellProcessor microfluidic chip, which contains a three-dimensional array of electrodes that allow users to design and control electrical-field configurations for cell manipulation.

"We developed the CellProcessor platforms for precise and fully automated sorting in a microfluidic environment," says Gabriele Gradl, Evotec's vice-president of cell handling and analysis. "The underlying technology makes cell analysis and isolation reproducible and predictable down to the single-cell level." The resulting platform allows for the delicate manipulation of small numbers of cells, in which the gentle handling provided by combining dielectrophoresis with hydrodynamic flow can be useful.

HOSH

M.E.



Inside Dako's MoFlo, which can sort up to 70,000 objects per second.

such commercial products, and remain a popular option. Miltenyi Biotec, based in Bergisch Gladbach, Germany, also offers reagents for performing column-based 'magnetic-assisted cell sorting' (MACS) using its MACS Separator. This is available in an automated version to simplify the purification process.

StemCell's offerings for magnetic separation include the EasySep system, which uses a specially designed high-gradient magnet to separate nanoparticle-labelled cells. These paramagnetic particles are particularly small, to an extent where they will not interfere with flow cytometry performed on purified cells. StemCell also offers an automated version, the RoboSep, which gives users a variety of options for configuring separation protocols.

Magnetic separation's usefulness is limited

by the inherent constraints on the number of sort parameters and its reliance on cell-surface antigens for sorting. Nonetheless, it excels at applications in which bulk separations need to be performed quickly, or as a prelude to more extensive cell-sorting procedures. "In a lot of cases for immunology research, the level of purity that you get with magnetic separation is more than adequate," says Woodside. "Basically, people want to do the fewest steps possible and get the purest cells back — that's what's driving this technology."

Bridging the gap

With all the interest in optimizing the efficiency and cost of cell sorting, it is understandable that James Leary, head of the molecular-cytometry facility at Purdue University in West Lafayette,

Indiana, is disappointed at the chilly reception that microfluidic platforms tend to receive in the community. "Flow cytometry has had microfluidics at its core for 40 years," he says. "But it's interesting, because the flow-cytometry groups and the microfluidics groups don't talk to each other very much. If they did, progress would be a whole lot faster."

Leary, a cell-sorting pioneer, is doing his part to bridge that gap through collaborations with colleagues such as Rashid Bashir, from his university's Birck Nanotechnology Center. The two see great advantages at the microscale, including portability, disposability and improved biosafety for handling pathogenic samples. They are exploring next-generation sorting technologies such as dielectrophoresis, in which a nonuniform electrical field is used to separate charge-neutral objects based on their size or chemical properties (see 'Playing the field', page 1181). "The dielectrophoretic approach is attractive because it is electrical, it is integrated and you don't have to have lots of mechanical valves," explains Bashir. "Moving the cells, instead of the fluid, makes more sense."

The use of laser light for cell manipulation is well-established (see 'The guiding light', below), and lasers are now being exploited for cell sorting. Kishan Dholakia, leader of the optical-trapping group at the University of St Andrews, UK, recently developed a system in which two- or three-dimensional patterns are generated by an optical-tweezers laser to create a 'passive' cell-sorting matrix. "We put cell samples on to an optical corrugation or

THE GUIDING LIGHT

Never mind Star Wars, lasers are good for more than burning and cutting, and in fact can be surprisingly gentle. Take 'optical tweezers', for example — a laser focused on a microscopic object creates an optical force trap that enables the precise manipulation of that object within a three-dimensional space.

Since their development in 1986, optical tweezers have generated a veritable bounty of valuable information, including insights into the physical properties of DNA molecules and the fundamental mechanisms of various enzymes and molecular motors.

They have also shown considerable promise for cell manipulation, although biologists have yet to fully explore their potential. "We have to educate people and make them comfortable with optical technology," says Kishan Dholakia, who heads the optical-trapping

group at the University of St Andrews, UK.

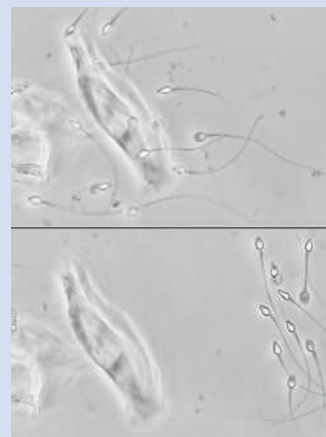
Many current users build their systems from scratch — a daunting and expensive prospect for the optics novice. To remedy this, Dholakia's team has lent its expertise to develop an entry-level, single-beam optical-tweezers workstation — the E3100 — for biologists looking to get their feet wet. The E3100 is available from Elliot Scientific in Harpenden, UK.

Cell Robotics of Albuquerque, New Mexico, also offers an off-the-shelf system: LaserTweezers, an adjustable single-trap system that is designed for integration with a standard inverted microscope, and which can also be incorporated as a module of a larger workstation to allow computerized control and full automation.

Single-beam traps enable impressive experiments, but the future clearly lies in higher-throughput platforms. "The

technology has always been associated with one to ten particles," says Dholakia. "I would like to see thousands of particles being ordered and sorted in a really rapid fashion."

Enter Arryx of Chicago, Illinois; its BioRyx 200 system uses holographic technology to



Caught: sperm cells isolated from epithelial cells using the BioRyx 200.

expand the number of tweezers simultaneously available to users. "It can generate up to 200 traps in a three-dimensional working volume, each of which is independently movable in real-time," says chief technology officer Dan Mueth. "You can pull on cells and sense or measure how they stretch, grab cells and move them around, probe the adhesion of cells, position cells for investigation, or isolate cells."

The BioRyx 200 also offers a software interface that enables real-time manipulation or automation of the traps. But multiple traps are not the only benefit of holographic optical trapping. "You can select from a variety of trap shapes with different properties," says Mueth. "There are plenty of other advantages that are more subtle but can have an impact, and have to do with optimizing performance and the ability to work with particular samples." M.E.

ARRYX

naturejobs

**THE CAREERS
MAGAZINE FOR
SCIENTISTS**

If a plan by the UK government to reshuffle research funds goes ahead, it could trigger an exodus of both pounds and professors from some of the country's most established universities in favour of a host of up-and-comers. Only two of the country's more prominent universities — the University of Oxford and University College London — stand to gain (see *Nature* **441**, 917; 2006) under the new plan. Other high-profile universities, such as the University of Manchester, Imperial College London and the University of Cambridge, would all lose significant funds and, therefore, research infrastructure and jobs.

The revamped method for distributing funds, which is tentatively scheduled to be phased in after 2008, is based on how much income universities already attract from competitive grants, charities and industrial contracts. Although the new benchmarks may still be used to compare funding distribution against the current Research Assessment Exercise (RAE), many researchers are concerned that the proposed system will cause major problems. Critics say that the system would bias funding towards applied over basic research and that it ignores important factors, such as the number of PhD students trained by an establishment, its publication record and the number of patents it has been granted.

The plan, which could greatly benefit some of Britain's less-heralded institutions, such as Cranfield University and the University of Greenwich, reminds me of what would happen if pleas by US members of Congress to reallocate money to underdog states such as Mississippi and Alabama were to be heeded. Yes, there would be a more even distribution of money, but it would be on a questionable basis and would give uncertain results. Maybe Britain needs to go back to the drawing board to find a way to give funds to researchers, rather than their institutions, based on the merit of the proposals and the individual scientists' track records. If not, some of the country's best researchers may not switch from Cambridge to Cranfield, as some who back the new plan might hope, but from Manchester to Massachusetts.

Paul Smaglik, *Naturejobs* editor

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MOVERS

**Gary Borisy, director and chief executive,
Marine Biological Laboratory,
Woods Hole, Massachusetts**



2003-06: Associate vice-president for research, Northwestern University, Evanston, Illinois

2000-06 Leslie B. Arey professor of cellular and molecular biology, Northwestern University, Illinois

1968-2000 Professor of molecular biology and zoology, University of Wisconsin, Madison

Gary Borisy's career has been propelled by motion. When the University of Chicago biochemistry major realized no one knew how chromosomes move during mitosis, he embarked on a lifelong quest to document basic cellular movements. Little did he know that his scientific journey would bring him repeatedly to the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts.

Doing his PhD research at Chicago, Borisy discovered that tubulin, the protein that makes up microtubules, is key to the cell division process. But it was an MBL summer course that spurred him to unlock more cell secrets. He learned to isolate the fibres, called mitotic spindles, that distribute chromosomes during cell division. "Once you work a summer in Woods Hole, you want to come back," he says of the interactive, camp-like escape for scientists.

Mentors, however, advised him to explore other areas. Hugh Huxley, a molecular biologist at the UK Medical Research Council in Cambridge who had worked on the mechanism of muscle contraction, welcomed Borisy as a postdoc. The time he spent in Huxley's lab gave Borisy a solid footing in the discipline of structural biology.

Borisy then went to the University of Wisconsin in Madison, but managed to maintain a summer laboratory at Woods Hole for five years to study cytoskeleton formation.

After a one-year sabbatical back at Cambridge, Borisy shifted his approach from the test tube to the cell. He went to the Weizmann Institute of Science in Rehovot, Israel, to collaborate with pioneers of the then-nascent fluorescent techniques combined with subcellular imaging. "That was a major turning point for me," says Borisy. A decade of *in vivo* cell-division work included documenting how cells crawl.

Next, Borisy went to Northwestern University, where he was soon made associate vice-president of research. The rave reviews he won for assembling a coalition of four Chicago-area institutions to advance health technologies led an MBL search committee to appoint him as director and chief executive. At first reluctant to be a full-time administrator, Borisy was swayed by his affection for MBL, according to John Dowling, president of the MBL corporation.

Borisy now looks forward to drawing on MBL's strengths in ecosystems, microbial evolution and cell dynamics, while enhancing the educational and collaborative opportunities. In doing so, he'll be responsible for maintaining the admin-free environment he's enjoyed for so many years — one of the qualities that makes MBL a scientific Shangri-La. ■
Virginia Gewin

SCIENTISTS & SOCIETIES

Networks work

When I chose to do a PhD project in computational biology, my supervisor balked. "Do you really want to work for three years on a computer?" he asked. He'd made a valid point: I needed to assess the best way to shape my path. I decided a multidisciplinary preparation was a must. I was soon to learn that international organizations of students and researchers can provide a valuable network for PhD project needs.

Given that my project focuses on the structure-function relationship in proteins, I needed experience with the wet-lab stuff of biochemistry and X-ray crystallography. Ideally, this would help me attain a broader vision and a better understanding of the structures and functions of interest to me.

I started to explore the possibility of becoming a visiting scientist outside Italy, by looking through the usual job ads. I thought it would be easy to find something. I wasn't expecting a salary, only a place receptive to hosting an enthusiastic Italian trainee in protein biochemistry. But I sent out countless CV and referee letters with no result.

My quest changed dramatically when I started to consider it from another perspective: any boss would rather find an employee through a personal contact than by e-mail. Marco Quarta, chairman of the Young European Biotech Network (YEBN), of

which I'm a member, advised me to send a message to members abroad, who would post it to their local networks.

I was intrigued. Although I chaired a task force for the YEBN, and recognized the value of its networking resources for teaching skills such as teamwork and project management, I hadn't thought of using it this way. Yet this group of professionals with different scientific backgrounds offered the potential for interesting collaborations.

Just one day after my posting I got an interesting proposal. If I had considered the 'network opportunity' earlier I would have saved some precious time.

I spent nine months at the University of Limerick in Ireland, in a biochemistry laboratory where the activities ranged from the identification and expression of promising genes encoding bacterial proteases and inhibitors, to the crystallization and computational work necessary to study proteins. Now I'm back in Milan in my home lab.

My time in Ireland was invaluable. I studied the practical issues behind protein structure determination and characterization. I know that my time there enriched my PhD experience and provided me with an additional skill set that will aid my career. ■

Denis Bilotta is a molecular-modelling PhD student at the University of Milano-Bicocca, Italy.

GRADUATE JOURNAL

Gooooaalll! A PhD!

These are exciting times for a scientist. On the day the Netherlands, my home country, defeated Serbia and Montenegro in the World Cup, I wrote two-thirds of the 'results' section of a paper. Both were major stepping-stones.

I've been spending days away from the lab in order to write the two papers that will form the heart of my thesis. In between paragraphs, I'm also revelling in the opening-rounds drama of the World Cup. Four years ago, the World Cup didn't present nearly so much of a distraction. To begin with, Holland wasn't playing. Moreover, I was a young graduate student lacking any sense of urgency. A few mornings away from the lab weren't going to change the outcome of my PhD.

This time around, I have deadlines to meet. So — except for taking two hours off to put on my orange wig, trot down to the pub with the other Europeans in town and root for Holland on match days — I have to content myself with following the games on my laptop while I write.

My PhD work has been intense. Not every day has the drama of a championship match, but with the final stage of my research in sight it's time to step it up. This month is the culmination of four years of preparation — for the Netherlands, who are about to face the second round as I write, and for me, who needs to commit a few more experiments to paper to form a thesis. Wish us both luck. ■

Milan de Vries is a molecular-biology graduate student at the Massachusetts Institute of Technology.

Finding a happy medium

Be careful what you think out loud...

John Gilbey

The lab meeting droned on through the blazing, fetid April afternoon. Air conditioning is for systems, not people, according to Tim — my department head — whose plodding tones mapped out a tale of woe and crisis.

"So that wraps it up for finance — it ain't good. But before you rush off", he raised his voice over the scrape of chairs, "I've got the latest hot news from the faculty board — so sit down. Thanks".

Pushing aside his notes, Tim laid his hands flat on the smeared glass of the table. "I don't need to remind you how much trouble the university has had with fraud over the past few years. I'm sure you all remember how hairy things got when Fred's team got busted for running two information systems — the real one and the one the journal referees got to see. And don't think I've forgotten how jealous some of you were — until they got rumbled..."

"Well, our beloved University of Rural England has been quietly rebuilding Internal Audit in order to, and I quote, 'bring a new level of assurance to our sponsors regarding scientific probity — through the innovative use of A-Psi technology.'"

"What the hell is A-Psi?" demanded the bald hippy beside me. Tim looked amused but embarrassed.

"Apparently it stands for 'Assisted Psychic'. The new auditor is a certified clairvoyante calibrated to international standards — and she's a biologist too, so don't think you'll be able to doubletalk your way out. 'Assisted' means they use some new gizmo to amplify and record the data stream while she reads your mind." Tim looked slowly round the table, "I can't say I envy her — because you lot are top of the list."

The meeting dissolved in uproar as Tim's MeetingSec gave up the unequal struggle to transcribe 12 streams of screamed invective.

By Monday, people had simmered down a bit, but how should we handle the audit? I asked Tim for advice. "Just one thing," he muttered, "try not to get her angry."

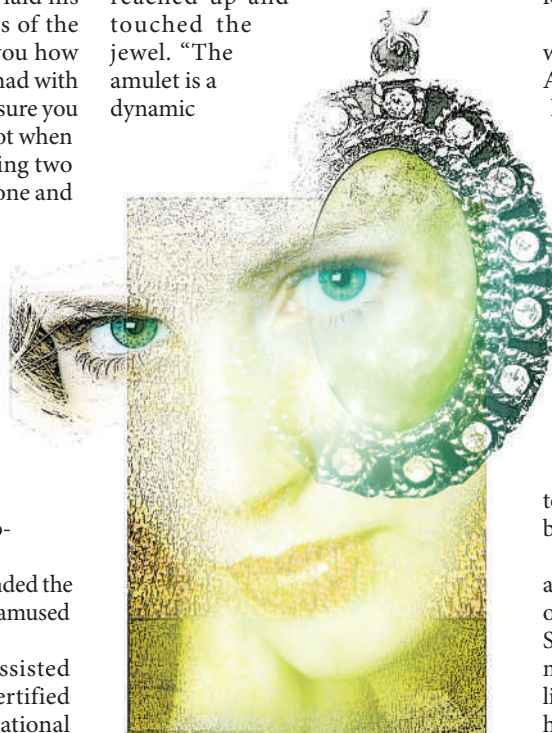
"Why not? What have you heard?"

Tim shook his head slowly. "Just don't, that's all."

I was pondering this when the audit

team arrived in my office. The lead auditor was tall, with long auburn hair and green eyes so piercing they looked like fakes. Somehow, I knew they weren't. Her companion was male, obese, sweaty and clutching a data recorder. She introduced him as Keith. I didn't take to Keith. Her name was Claire — which I tried hard not to find amusing, given Tim's advice.

Over her black business suit Claire wore a pendant set with a stone that looked something like opal — patterns of light flashed from it as she moved. Her hand reached up and touched the jewel. "The amulet is a dynamic



nanotech array," she explained. "It helps me see the way you react to things."

"You mean to read my mind?" I blurted out.

"No! Why does everyone think that? I can only see what you choose to show me, only that!" The colour rose in Claire's cheeks, and I waited for a further explosion — but she composed herself and continued in measured tones.

"Good morning, John, this is audit session URE/BIO/009, 23 April 2026. My terms of reference for this audit are as follows..." Keith's recorder began to hum quietly.

Two hours later things were winding up. "OK, John, we've looked at data management and the document system. I am happy with the sample schedule and we've

agreed some actions regarding the management of links to the EMS."

Claire looked straight into my eyes. Keith tapped some inscrutable command into the recorder. "Now, is there anything else you want to tell me? Anything at all?"

Claire lowered her head slightly, keeping me fixed in her gaze. "This is really important to the outcome of the audit..."

I felt guilty and slightly dizzy — but shook my head. Then it was all over. Keith prodded his recorder again, and they were gone — with the draft report promised for Friday.

Tim — inevitably — wasn't pleased when the report offered only "Limited Assurance" of my work. "That means," he pointed out, "they think you're hiding something. They want you back on Monday — just go down there and give them whatever they ask for."

After a weekend of stomach-churning uncertainty, I presented myself at the appointed door in the Registry building. I knocked: it opened. Gently filtered light suffused the room. Claire was sitting on a sofa, holding the amulet.

She motioned me to sit down. An interactive hologram of a ginger cat turned at my approach, then went back to sleep. Keith, thankfully, was nowhere to be seen.

The question came again — "Is there anything you want to tell me?" I tried, without success, to stem the rush of thoughts. Scenes of unquenchable desire — Claire, myself and our future together — burst out like water from a broken dam, to my utter horror and bewilderment. I began to stutter an apology, but she silenced me with a single flash of green.

She gently placed my hand over the amulet. It felt like the first touch of morphine — like having my brain rinsed in cool spring water. Then came a rush of images from Claire, almost a mirror of my own but deeper, more powerful and dramatically more graphic — along with a pang of loneliness.

"How did you do that?" I whispered.

Claire smiled and closed her hand over mine, pressing the amulet into my palm. "Let's just say it's an undocumented feature..." The cat stretched languidly and began to purr.

John Gilbey is a hopeless romantic, or just hopeless — depending on your point of view. He is keen to point out that he writes in a private capacity.

JACEY